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Título de la tesis: Disolventes Supramoleculares funcionales utilizando agua como agente coacervante: modelado, caracterización y aplicaciones analíticas// Water-induced functional Supramolecular Solvents: modeling, characterization and analytical applications

Directores: Soledad Rubio Bravo y Diego García Gómez

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TITULO: *Water-induced functional Supramolecular Solvents: modeling, characterization and analytical applications*

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UNIVERSIDAD D CÓRDOBA

Departamento de
Química Analítica

TESIS DOCTORAL

Disolventes Supramoleculares funcionales utilizando agua como agente coacervante: modelado, caracterización y aplicaciones analíticas

Water-induced functional Supramolecular Solvents: modeling, characterization and analytical applications

JOSÉ ÁNGEL SALATTI DORADO

Tesis Doctoral:

Disolventes Supramoleculares funcionales utilizando agua como agente coacervante: modelado, caracterización y aplicaciones analíticas

Trabajo presentado, para optar al grado de Doctor, por

José Ángel Salatti Dorado

que lo firma en Córdoba, 1 de noviembre de 2018

Firmado:

José Ángel Salatti Dorado

Licenciado en Ciencias Ambientales

con el VºBº de los directores,
Soledad Rubio Bravo y Diego García Gómez

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Profesor Ayudande Doctor del Departamento de Química Analítica de la Universidad de Salamanca

Mediante la defensa de esta memoria se opta a la obtención de la mención de "Doctorado Internacional" habida cuenta de que el doctorando reúne los requisitos exigidos:

1. El doctorando ha realizado una estancia de 3 meses en el "*Institut National de la Santé et de la Recherche Médicale Unit 1148*" (Francia), que ha contribuido a su formación.
2. Parte de la Tesis Doctoral, en concreto el objeto, el contenido, la introducción y las conclusiones, se han redactado en una de las lenguas oficiales de la Unión Europea distinta a cualquiera de las lenguas oficiales en España.
3. Cuenta con informes favorables de dos doctores pertenecientes a instituciones de enseñanza superior o institutos de investigación de países europeos distintos a España.
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Soledad Rubio Bravo, catedrática del Departamento de Química Analítica de la Universidad de Córdoba y **Diego García Gómez**, profesor ayudante doctor del Departamento de Química Analítica de la Universidad de Salamanca en calidad de directores de la Tesis Doctoral presentada por el licenciado en Ciencias Ambientales, Dº. José Ángel Salatti Dorado,

CERTIFICAN: Que la citada Tesis Doctoral "Síntesis y caracterización de sistemas supramoleculares constituidos por agregados estables. Aplicación en procesos de extracción analítica" se ha realizado en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba y que, a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajos.

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Dr. Diego García Gómez

Profesor Ayudande Doctor del Departamento de Química Analítica de la Universidad de Salamanca

Agradezco al Ministerio de Economía y Competitividad (MINECO) la concesión de una ayuda de Formación de Profesorado Universitario con referencia FPU13/03796, que me ha facilitado la plena dedicación a esta Tesis Doctoral, así como la concesión de una ayuda complementaria para beneficiarios de ayudas FPU: Estancias Breves y Traslados Temporales 2016 con referencia EST16/00685.

TÍTULO DE LA TESIS:

Disolventes Supramoleculares funcionales utilizando agua como agente coacervante: modelado, caracterización y aplicaciones analíticas

DOCTORANDO: José Ángel Salatti Dorado

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Las investigaciones desarrolladas en esta Tesis Doctoral han tenido como objetivo general la síntesis, caracterización y aplicación de disolventes supramoleculares (SUPRAS) diseñados para aportar soluciones concretas en dos ámbitos en los que los procesos de extracción son de gran relevancia; el tratamiento de muestras para el análisis químico de contaminantes, y la recuperación de sustancias bioactivas a partir de biomasa vegetal para aplicación en la industria alimentaria.

Para ello se han diseñado y/o aplicado SUPRAS con las siguientes funcionalidades:

1. Capacidad para la extracción eficiente de contaminantes y producción de extractos que no originen efectos matriz en el análisis de muestras biológicas mediante LC-MS/MS.
2. Capacidad para reducir o eliminar la pérdida de compuesto anfifílico en la disolución de equilibrio para aplicación al tratamiento de aguas residuales y el análisis químico de contaminantes en elevados volúmenes de muestra.
3. Elevada estabilidad térmica para aplicación en análisis mediante cromatografía de gases-espectrometría de masas.
4. Capacidad de extraer, estabilizar y encapsular sustancias bioactivas con el fin de transportar las mismas desde la biomasa vegetal hasta el alimento.

Para obtener estas funcionalidades se han sintetizado SUPRAS mediante fenómenos de autoensamblaje y coacervación a partir de los siguientes compuestos anfifílicos: hexanol, ácido poli-undecenoico y ácido octanoico. En todos los casos, se ha utilizado agua como agente coacervante.

Las aplicaciones más relevantes en las investigaciones desarrolladas incluyen la determinación de bisfenol A en orina, 37 disolventes residuales en formulaciones farmacéuticas y la integración de las etapas de extracción y encapsulamiento de astaxantina para su utilización como aditivo alimentario.

Los resultados de las investigaciones realizadas se han materializado en 4 artículos científicos (3 publicados en revistas científicas indexadas situadas en el primer cuartil y 1 enviado a publicación). Asimismo, el doctorando ha participado en la publicación de 1 artículo científico (Molecules 2018, 23 (10), 2601, Q2). Los resultados obtenidos se han presentado por el doctorando en 5 contribuciones orales a congresos (4 nacionales y 1 internacional) y 4 carteles nacionales.

En base a la originalidad de las investigaciones desarrolladas y expuestas en esta Memoria así como la formación científica adquirida por D. José Ángel Salatti Dorado, autorizamos la presentación de esta Tesis Doctoral.

Córdoba, 1 de noviembre de 2018

Firma de los directores

Fdo.: Soledad Rubio Bravo

Fdo.: Diego García Gómez

MIS AGRADECIMIENTOS:

En este hermoso a la par que arduo camino que es la Tesis Doctoral siempre he tenido la gran suerte de estar acompañado en cada momento de gente a la que quiero y que me ha apoyado en cada instante que lo he necesitado.
Han sido cinco años en los cuales la vida me ha permitido no solo evolucionar como ser humano sino el poder compartir dicha evolución con aquellas personas a las cuales quiero agradecer todo lo que han hecho por mí para poder llegar a este día.

En primer lugar, agradezco a mis padres por todo el apoyo brindado durante estos años, por estar ahí siempre que los he necesitado y por ser un pilar fundamental en mi vida. Sin ellos no estaría donde hoy estoy ya que son ellos los que me han levantado en mis caídas que no han sido pocas y me han podido regalar lo más importante en este mundo que es la vida. Gracias por ser como sois, gracias por darme la oportunidad de ser alguien en la vida, gracias por hacer este sueño posible y por brindarme los valores que hoy tengo y han hecho que cada día me levante con la premisa de que si lo crees puedes. Os quiero.

Mención especial en estos agradecimientos se merece una persona que ha sido, es y será un hermano para mí. Angelillo ha sido mi consejero, mi confidente, elevó la palabra amistad a un nivel insuperable y no hay vida suficiente para poder agradecerle todo lo que ha hecho porque el día de hoy sea posible. Siempre estaré a tu lado apoyándote como has hecho tu cada día y todos tus éxitos personales y profesionales me harán igual de feliz que los míos propios.

En 2016 el destino me permitió verte por primera vez y su capricho me hizo conocerte un año después. Me has acompañado en muchos momentos aun siendo miles de kilómetros los que nos separaban. En 2018 se me ha brindado la oportunidad de compartir un momento muy importante de mi vida a tu lado haciendo que cada día sea único. Sofi, te admiro como profesional, te adoro como persona, te quiero y te querré de todas las maneras posibles. Eres, has sido y serás el mayor apoyo que tengo en mi vida. Gracias por estar a mi lado.

Tengo unos amigos que valen oro. Unas personas increíbles que durante esta etapa me han acompañado y aguantado en mis peores momentos y aun así han seguido a mi lado dándole valor a la palabra amistad. Por estas personas estoy aquí ya que sin ellas este camino no hubiera sido posible. Os quiero mucho y de corazón espero devolveros algún día todo lo que me habéis dado. Este espacio de mi corazón va dedicado a vosotros Rafa (Choca), Vega, Antonio, Julián, Juan Carlos, Tomás, Márquez, Amparo, mi cuñi Ana, Isa, Lucía y mis "compis" Ana Ballesteros, Carmen y Encarni.

La familia para mí siempre ha sido un pilar fundamental en mi vida. No siempre tiene que haber lazos de sangre para que los consideres así. Es por ello que quiero agradecer a la familia Vargas Merina porque no siendo familia carnal si lo son de corazón que es lo más importante en esta vida. Gracias por todos los momentos disfrutados.

Mi nombre es mi esencia, todos me conocen así, este apellido es algo más que unas letras, es una forma de ser, de sentir, de agradecer. Por eso estas palabras van dedicadas a la Familia Salatti por ser como son y por estar siempre sacando una sonrisa en cada momento de la vida. Me gustaría destacar dentro de esta familia a una mujer que ha sido como una madre para mí y que hizo cientos de kilómetros para ayudar en los peores momentos que pasé en esta vida. Tita Rosa, eres un ejemplo a seguir como persona y te admiro por ello, nunca tendré tiempo de agradecerte todo lo que has hecho por mí y mis padres. Te quiero mucho.

Tras once años no todo el mundo puede decir que empezó a estudiar una carrera universitaria. Tras once años no todo el mundo puede decir que encontró en esa carrera una nueva familia, no todo el mundo puede decir tras once años que la familia sigue unida. Muchas gracias a la "Familia" ambiental (Loles, Isa, Silvia, Almudena, Sandra, Belén, Félix, Javi, Angelote, Víctor…) los cuales hemos compartido y seguimos compartiendo grandes momentos demostrando que la carrera es la mejor etapa de nuestra vida.

A mis perros Rayo, Trosky, Chico y Tolín porque han sido, son y serán por siempre lo mejor que me ha pasado en la vida. Gracias a ellos superé grandes problemas y siempre han estado ami lado para sacarme una sonrisa en cada momento. Esta Tesis es también vuestra y siempre os querré.

Mención especial a un grupo "random" que tenían como bandera el disfrutar y simplemente ser felices en cada momento que se veían para compartir experiencias investigadoras. Este grupo de grandes amigos les agradezco el ayudarme a ver la Tesis de otra manera. Muchas gracias a Bea, Julia, Marisa, Miguel, Javi y Fidel porque aún nos quedan muchos momentos por disfrutar juntos.

A mis compañeros de laboratorio (Noelia, Francesca, Sole, Guille, Aleja y María Jesús) ya que con ellos he compartido la mayoría de esta etapa y sin ellos no hubiera sido igual. Hemos pasado grandes momentos dentro y fuera del laboratorio y gracias a vosotros he aprendido mucho. Esté donde esté siempre me acordaré de lo feliz que he sido a vuestro lado.

Fueron tres meses solamente pero solamente con ellos pude descubrir a tres mujeres que admiro, respeto y quiero. Muchas gracias Graciela, Virginie y Violeta por hacer que mi estancia en París fuera como estar en mi hogar, por hacerme feliz, por enseñarme y ayudarme tanto. Gracias a la vida por permitirme haber compartido y seguir compartiendo tanto con vosotras.

Un agradecimiento más que especial es a una mujer que en 2008 hizo que empezara a amar la ciencia. Una mujer que a pesar de todo siempre estuvo ahí y cuando más la necesitaba más me apoyó. Su calidad como investigadora solamente es superada por su inmenso corazón y por la gran persona que es. En muchas ocasiones y sin necesidad ejerció como una madre y siempre estaré agradecido a ella. Muchas gracias Sole.

A mi co-director Diego le agradezco porque ha sido la persona que me hizo volver a creer en mí.

Por último, agradecer a aquellas personas que me dejaron en el camino, pero aun en la distancia siguen cuidándome desde el cielo (Abuelos, Tata, Peque, Tito Manolo y Rafael Cantón). Muchas gracias por haber estado en vida a mi lado y por acompañarme en la distancia hasta llegar al día de hoy.

Al final todo saldrá bien y si no sale bien,

es que aún no es el final

ÍNDICE

OBJETO

El desarrollo y aplicación de disolventes alternativos a los disolventes orgánicos en procesos de extracción analítica e industrial, con la finalidad de incrementar la selectividad y rendimiento de los mismos y reducir los costes e impacto ambiental asociados, es un reto al que muchos investigadores están dedicando gran esfuerzo. En este contexto, existe creciente interés por el diseño y síntesis de disolventes respetuosos con el medio ambiente que posean características programadas para cumplir funciones específicas.

Las investigaciones desarrolladas en esta Tesis Doctoral han tenido como objetivo la síntesis, caracterización y aplicación de disolventes supramoleculares (SUPRAS) diseñados para aportar soluciones concretas en dos ámbitos en los que los procesos de extracción son de gran relevancia; el tratamiento de muestras para el análisis químico de contaminantes, y la recuperación de sustancias bioactivas a partir de biomasa vegetal para aplicación en la industria alimentaria.

Los SUPRAS presentan propiedades de solubilización y operacionales idóneas para aplicación en procesos de extracción. Por otro lado, el hecho de que se sinteticen mediante fenómenos de autoensamblaje y que la composición y propiedades del SUPRAS puedan modelarse mediante la selección del compuesto anfifilico y el ambiente en el que se produce el autoensamblaje, ofrece multiples oportunidades para el desarrollo de disolventes funcionales.

Los objetivos específicos de las investigaciones realizadas en esta Tesis Doctoral, así como las funcionalidades programadas para los SUPRAS desarrollados, se especifican a continuación.

Objetivo 1

Desarrollo de SUPRAS con capacidad para la extracción eficiente de contaminantes y producción de extractos que no originen efectos de matriz en LC-MS/MS.

Antecedentes. Nuestro grupo de investigación (Química Analítica Supramolecular, SAC) ha desarrollado SUPRAS con propiedades de acceso restringido, basados en alcanoles con longitud de cadena hidrocarbonada en el intervalo C8-C14, que eliminan

la interferencia de proteínas y carbohidratos mediante mecanismos químicos y físicos, respectivamente.

Funcionalidad programada. Se investigará la formación de SUPRAS con alcanoles volátiles con el objetivo de no sólo excluir proteínas y carbohidratos en el proceso de extracción sino también eliminar la fracción lipídica y el alcanol.

Objetivo 2

Desarrollo de SUPRAS en los que se minimice la pérdida de compuesto anfifílico en la disolución de equilibrio para aplicación al tratamiento de aguas residuales y el análisis químico de contaminantes en elevados volúmenes de muestra.

Antecedentes. El grupo de investigación SAC ha desarrollado SUPRAS vesiculares con elevada estabilidad cinética en los que la concentración de compuesto anfifílico disminuye considerablemente con respecto a la concentración de agregación crítica (cac) usualmente presente en la disolución de equilibrio con el SUPRAS.

Funcionalidad programada. Se investigará la formación de SUPRAS con oligómeros anfifílicos ya que se ha demostrado que la cac disminuye progresivamente en función del número de monómeros que constituye el oligómero.

Objetivo 3

Desarrollo de SUPRAS con elevada estabilidad térmica que proporcionen extractos compatibles con GC-MS.

Antecedentes. Aunque SUPRAS se han combinado con GC-MS, el tensioactivo que constituye el disolvente debe eliminarse del extracto o derivatizarse previo al análisis del mismo.

Funcionalidad programada. Se investigará la formación de SUPRAS con oligómeros anfifílicos que presenten un elevado punto de ebullición y por tanto sean compatibles para su uso en espacio de cabeza-GC-MS.

Objetivo 4

Desarrollo de SUPRAS multifuncionales con capacidad de transportar sustancias bioactivas desde la biomasa vegetal hasta el alimento.

Antecedentes. El grupo SAC ha aplicado SUPRAS para la extracción de sustancias bioactivas a partir de microalgas y residuos agroindustriales.

Funcionalidad programada. Se investigará la aplicación de SUPRAS para ejercer múltiples funciones: extracción de sustancias bioactivas, estabilización de sus propiedades y encapsulamiento de las mismas.

Un objetivo transversal en la realización de la tesis doctoral ha sido la formación del doctorando a través de actividades complementarias a la labor investigadora, tales como la redacción de artículos científicos, la asistencia y presentación de comunicaciones en congresos nacionales e internacionales, la discusión crítica de los resultados, etc.

OBJECT

The development and application of alternative solvents to replace organic solvents in analytical and industrial extraction processes, in order to increase their selectivity and performance and reduce their costs and environmental impact, is a challenge to which many researchers are devoting efforts. In this context, there is a growing interest in the design and synthesis of environmentally friendly solvents that should show characteristics that allow to fulfill specific functions.

The research developed in this Doctoral Thesis has aimed at the synthesis, characterization and application of supramolecular solvents (SUPRAS) designed to provide specific solutions in two areas in which extraction processes are of great relevance: sample treatment for chemical analysis of contaminants and recovery of bioactive substances from vegetable biomass and their subsequent application in the food industry.

SUPRAS show ideal solubilization and operational properties for their application in extraction processes. Furthermore, they are synthesized by means of self-assembly phenomena and their composition and properties can be modeled by selecting the amphiphilic compound and the environment in which the self-assembly occurs, which offers multiple opportunities for the development of functional solvents.

The specific objectives of the research carried out in this Doctoral Thesis, as well as the functionalities programmed for the SUPRAS developed, are listed below.

Objective 1

Development of SUPRAS for the efficient extraction of contaminants yielding extracts able to avoid matrix effects in LC-MS/MS.

Background. Our research group (Supramolecular Analytical Chemistry, SAC) has developed SUPRAS with restricted access properties, based on alkanols with hydrocarbon chain lengths in the C8-C14 range, which remove the interference of proteins and carbohydrates through chemical and physical mechanisms, respectively.

Expected functionality. The synthesis of SUPRAS from volatile alkanols will be investigated in order not only to exclude proteins and carbohydrates in the extraction process but also to remove the lipid fraction and the alkanol.

Objective 2

Development of SUPRAS in which the loss of the amphiphilic compound in the equilibrium solution is minimized and their application to wastewater treatment and the chemical analysis of contaminants in high-volume samples.

Background. The SAC research group has developed vesicular SUPRAS with high kinetic stability in which the concentration of the amphiphilic compound in the equilibrium solution decreases significantly when compared to the critical aggregation concentration (cac) usually present in the equilibrium solution.

Expected functionality. The synthesis of SUPRAS from amphiphilic oligomers will be investigated since it has been demonstrated that the cac decreases progressively in a positive relation with the number of monomers that form the oligomer.

Objective 3

Development of SUPRAS with high thermal stability that result in extracts compatible with GC-MS.

Background. Even though SUPRAS have been previously combined with GC-MS, all these combinations involve the surfactant being removed from the extract or derivatized prior to the GC analysis.

Expected functionality. The synthesis of SUPRAS from amphiphilic oligomers with a high boiling point, and therefore compatible with headspace-GC-MS, will be investigated.

Objective 4

Development of multifunctional SUPRAS able to transfer bioactive substances from vegetable biomass to food.

Background. SUPRAS have been applied by the SAC group for the extraction of bioactive substances from microalgae and agroindustrial wastes.

Scheduled functionality. The application of SUPRAS to perform multiple functions, such as the extraction of bioactive substances, the stabilization of their properties and their encapsulation, will be investigated.

A key aim in this Thesis has been the development of a formation program for the PhD student through other activities apart from research, such as the writing of scientific papers, the attendance and presentation of contributions in national and international congresses, the critical discussion of the results, etc.

CONTENIDO

El contenido de la Memoria de esta Tesis Doctoral se ha estructurado en cuatro capítulos precedidos de una Introducción general en la que se discuten aspectos teóricos y prácticos relacionados con la síntesis, posibilidades de modelado y aplicación de los disolventes supramoleculares (SUPRAS) así como el encapsulamiento de sustancias bioactivas lipofílicas en transportadores lipídicos nanoestructurados. A continuación, se describen los contenidos de cada uno de los capítulos que recogen los resultados de las investigaciones realizadas.

Capítulo I: Disolventes supramoleculares constituidos por compuestos anfifílicos volátiles para reducción de los efectos matriz originados por fosfolípidos en LC-MS/MS

La investigación planteada en este capítulo se ha centrado en la síntesis y caracterización de SUPRAS volátiles con propiedades de acceso restringido para la eliminación de efectos matriz en el análisis de muestras biológicas mediante LC-MS/MS. Para ello se han generado SUPRAS a partir de disoluciones de hexanol en THF mediante la adición de agua, utilizada como agente coacervante. Se construye el diagrama de fases para la mezcla ternaria, donde se delimita la región de formación del SUPRAS, y se investiga la composición del mismo en función de las condiciones ambientales establecidas para el autoensamblaje de hexanol. Se establece la ecuación que predice el volumen de SUPRAS formado en función de las condiciones ambientales y se determina que, al igual que otros alcanoles, hexanol forma en el SUPRAS agregados hexagonales inversos en los que los grupos alcohol delimitan cavidades acuosas mientras las cadenas hidrocarbonadas están dispersas en THF.

La capacidad de los SUPRAS sintetizados para la eliminación de efectos matriz en bioanálisis se investiga mediante la determinación de bisfenol A (BPA) en orina. Se investiga la formación in situ del SUPRAS en esta matriz y la eliminación de proteínas y fosfolípidos. Se demuestra que los extractos supramoleculares pueden analizarse directamente mediante LC-MS/MS sin efectos matriz, lo que posibilita que la cuantificación de BPA pueda realizarse mediante calibración externa. BPA se extrae cuantitativamente en el SUPRAS (recuperaciones en el intervalo 96-107%). El límite de cuantificación del método para BPA es 0.025 ng/mL. El método se aplica a la cuantificación de BPA en muestras de orina.

Capítulo II: Disolventes supramoleculares constituidos por tensioactivos oligoméricos para reducir las pérdidas de tensioactivo en la disolución de equilibrio

Las investigaciones desarrolladas en este capítulo se han centrado en la síntesis y caracterización de SUPRAS obtenidos a partir de disoluciones del ácido poli-undecilénico (un tensioactivo oligomérico) en THF o etanol, utilizando agua como agente coacervante. Se ha realizado un estudio comparativo de ambas mezclas ternarias en términos de diagramas de fases, composición química de los SUPRAS generados en función de las condiciones ambientales, volumen de SUPRAS formado y características estructurales. Se han derivado diferentes ecuaciones para predicción del volumen y composición química de los SUPRAS generados en función de la composición de la disolución en la que se produce el autoensamblaje del oligómero. Se demuestra la hipótesis de que en la región de formación del SUPRAS, el oligómero se incorpora completamente al mismo, lo que posibilitará su aplicación al tratamiento de aguas residuales y al análisis de contaminantes cuando se requiera la utilización de elevados volúmenes de agua.

Capítulo III: Disolventes supramoleculares térmicamente estables para aplicación en cromatografía de gases con espacio de cabeza

Las investigaciones descritas en este capítulo se centran en la síntesis de SUPRAS a partir de disoluciones del oligómero ácido poli-undecilénico en tetraglima mediante la adición de agua como agente coacervante. Se construyó el diagrama de fases de la mezcla ternaria para delimitar la región de formación de los SUPRAS y estos se caracterizaron en términos de composición química y propiedades físicas tales como la estabilidad térmica.

La aplicabilidad de estos SUPRAS en cromatografía de gases de espacio de cabeza (HS-GC) se investigó mediante el análisis directo de los extractos obtenidos en la extracción de disolventes residuales en formulaciones farmacéuticas. La metodología desarrollada permitió la extracción cuantitativa de las diferentes clases de disolventes residuales legislados (37 disolventes pertenecientes a las clases 1, 2A, 2B y 2C) y su determinación mediante HS-GC-MS. La determinación de los disolventes de la clase 2C es muy compleja utilizando los procedimientos previamente descritos debido a los elevados puntos de ebullición de los mismos. Se demostró que el método es aplicable a

una gran variedad de formulaciones farmacéuticas y éste se validó de acuerdo a los criterios establecidos en la legislación [ICH Q2 R1].

Capítulo IV: Disolventes supramoleculares multifuncionales para combinar la extracción y encapsulación de componentes bioactivos lipofílicos

Las investigaciones descritas en este capítulo se han desarrollado en el *Institut National de la Santé et de la Recherche Médicale Unit 1148* en París, durante la estancia realizada por el doctorando en este centro. Estas investigaciones han tenido como objetivo la combinación de SUPRAS y transportadores lipídicos nanoestructurados (NLCs) para extraer, estabilizar y encapsular eficazmente compuestos bioactivos lipofílicos. Para ello, se han utilizado SUPRAS, previamente descritos por el grupo de investigación SAC, constituidos por ácido octanoico en medio etanol-agua que se han utilizado para la extracción de astaxantina a partir de *Haematococcus pluvialis*. En investigaciones previas, el grupo SAC ha demostrado que estos SUPRAS con propiedades de acceso restringido extraen cuantitativamente astaxantina y producen oleorresinas que tienen la misma composición que las obtenidas mediante extracción con fluidos supercríticos (SFE), pero a mucho menor coste. Las oleorresinas así obtenidas se utilizaron como la fracción lipídica líquida para la obtención de las SUPRAS-NLCs. Estas nanopartículas se caracterizaron mediante diferentes técnicas: DLS, AFM y Crio-SEM, demostrándose que se forman partículas esféricas con tamaño aproximado de 100 nm. Su actividad antioxidante se determinó mediante ORAC y α-TEAC y su capacidad para inhibir ROS in vitro se evaluó mediante la sonda DHE. Los resultados demostraron que las SUPRAS-NLCs presentan una estabilidad excepcional con respecto a investigaciones previas (al menos 6 meses a 4ºC).

En esta Memoria también se incluye un apartado en el que se discuten las principales conclusiones derivadas de los resultados obtenidos.

Finalmente, en el apéndice A se enumeran los artículos científicos publicados en revistas internacionales especializadas a los que ha dado lugar esta Tesis Doctoral y en el Apéndice B, las comunicaciones realizadas a Congresos nacionales e internacionales.

SUMMARY

The content of this Doctoral Thesis has been structured in four chapters preceded by a general Introduction in which the theoretical and practical aspects related to the synthesis, modeling possibilities and application of supramolecular solvents (SUPRAS) as well as the encapsulation of lipophilic bioactive substances in nanostructured lipid carriers are discussed. Below, the content of each chapter is described:

Chapter I: Volatile amphiphile-based supramolecular solvents for reducing phospholipid-based matrix effects in LC-MS/MS

The research presented in this chapter has focused on the synthesis and characterization of volatile SUPRAS with restricted access properties for the elimination of matrix effects in the analysis of biological samples by LC-MS/MS. For this purpose, a SUPRAS has been synthetized from hexanol in THF solutions by the addition of water as coacervating agent. The phase diagram for this ternary mixture was constructed, delimiting the SUPRAS region, and its composition was investigated according to the environmental conditions established for the self-assembly of hexanol. The equation predicting the volume of SUPRAS formed as a function of environmental conditions was established. As in the case of other alkanols, hexanol aggregates in the SUPRAS as inverse hexagonal structures in which the alcohol groups surround aqueous cavities while the hydrocarbon chains are dispersed in THF.

The ability of the SUPRAS synthesized for the removal of matrix effects in bioanalysis was investigated by the determination of bisphenol A (BPA) in urine. The *in-situ* formation of SUPRAS in this matrix and the removal of proteins and phospholipids were investigated. It was demonstrated that supramolecular extracts can be analyzed directly by LC-MS/MS avoiding matrix effects, which allowed the quantification of BPA via external calibration. BPA was quantitatively extracted in the SUPRAS (recoveries in the 96-107% range). The limit of quantification of the method for BPA was 0.025 ng/mL. The method was applied to the quantification of BPA in urine samples.

Chapter II: Supramolecular solvents formed by oligomeric surfactants to reduce surfactant losses in the equilibrium solution

The research carried out in this chapter has focused on the synthesis and characterization of SUPRAS obtained from solutions of poly-undecylenic acid (an oligomeric surfactant) in THF or ethanol, using water as coacervating agent. A comparative study of both ternary mixtures has been performed in terms of phase diagrams, the chemical composition of SUPRAS generated from different environmental conditions, the volume of SUPRAS formed and structural characteristics. Different equations have been derived, generated as a function of the composition of the synthetical solution, for predicting the volume and chemical composition of these SUPRAS. The hypothesis of the oligomer being completely incorporated into the SUPRAS was fully demonstrated. This property will make possible the application of this novel SUPRAS to wastewater treatment and to the analysis of pollutants when the use of high volumes of water is required.

Chapter III: High thermally stable supramolecular solvents applicable to headspace gas chromatography

The research described in this chapter is focused on the synthesis of SUPRAS from solutions of the oligomer poly-undecylenic acid in tetraglyme via the addition of water as coacervating agent. The phase diagram from this ternary mixture was constructed, delimiting the SUPRAS formation region, that was also characterized in terms of chemical composition and physical properties such as thermal stability.

The applicability of these SUPRAS in headspace gas chromatography (HS-GC) was investigated by the direct analysis of the extracts obtained from the extraction of residual solvents in pharmaceutical products. The methodology developed allowed the quantitative extraction of the different legislated classes of residual solvents (37 solvents belonging to classes 1, 2A, 2B and 2C) and their determination by HS-GC-MS. The determination of the 2C class solvents by previously described procedures is very complex due to their high boiling points. It was demonstrated that the method can be applied to a wide variety of pharmaceutical products. The method was also validated according to the criteria established in the relevant legislation [ICH Q2 R1].

Chapter IV: Multifunctional supramolecular solvents for the combination of the extraction and encapsulation of lipophilic bioactive components

The research described in this chapter has been developed in the "Institut National de la Santé et de la Recherche Médicale Unit 1148" in Paris, during the stay of the PhD student in this center. This research has aimed at the combination of SUPRAS and nanostructured lipid carriers (NLCs) for the effective extraction, stabilization and encapsulation of lipophilic bioactive compounds. For this purpose, SUPRAS previously described by the SAC research group for the extraction of astaxanthin from *Haematococcus pluvialis* and formed by octanoic acid in ethanol-water have been used. In previous research, the SAC group has demonstrated that these SUPRAS with restricted access properties quantitatively extract astaxanthin and are able to produce oleoresins that show the same composition as those obtained by extraction with supercritical fluids (SFE), but at a much lower cost. The oleoresins thus obtained were used as the liquid lipid fraction to get SUPRAS-NLCs. The synthetized nanoparticles were characterized by several different techniques: DLS, AFM and Cryo-SEM microscopy, indicating that spherical particles with an approximate size of 100 nm were formed. Their antioxidant activity was determined by ORAC and α-TEAC and its ability to inhibit ROS in vitro was assessed by the DHE probe. The results showed that the SUPRAS-NLCs here synthetized exhibit an exceptional stability (at least 6 months at 4ºC) when compared to previous research.

This Report also includes a section in which the main conclusions derived from the obtained results are discussed.

Finally, appendix A lists the scientific papers published in specialized international journals derived for this Thesis and, appendix B, the contributions made to national and international conferences.

INTRODUCTION

1. Supramolecular Analytical Chemistry

1.1. General aspects

Supramolecular Chemistry is the branch of chemistry that focuses on the study of entities formed by molecules or ions through noncovalent interactions such as electrostatic, dispersion, dipole-dipole, hydrogen bonds and solvophobic effects. Supramolecular chemistry is a dynamic chemistry, in view of the lability of the interactions that connect the molecular components of a supramolecular entity and the resulting capacity of supramolecular species to exchange their constituents in order to develop highly complex chemical systems [1]. Supramolecular chemistry has paved the way for the implementation of the concept of molecular information in chemistry, with the aim of obtaining a progressive control over the spatial (structural) and temporal (dynamic) characteristics of the matter and its complexity through self-organization [2–4].

Like all emerging sciences, Supramolecular Chemistry has been born being a multidisciplinary science [5,6]. There are three interconnected fundamental areas that must be considered in the study of Supramolecular Chemistry that are the basis for the knowledge and design of the organization of the matter in progressively more complex systems [2]. The first one is that of molecular recognition and the fundamentals derived from it (reactivity, catalysis and transport) [4,7]. The second one refers to self-assembly and self-organization and it is based on the design and application of programmed systems. The third and the most emergent one introduces the concepts of adaptation to the environment and evolution, which allow the design of adaptive, programmed and dynamic chemical systems. This last level belongs to the Dynamic Constitutional Supramolecular Chemistry (CDC) [1,3,8–10].

Supramolecular chemistry is, by nature, a dynamic chemistry and therefore, reversible due to the different associations between its components that allows a continuous change in the constitution of the entity [11].

Nowadays, the applications of Supramolecular Chemistry cover every one of the known scientific disciplines. It should be noted its use in the area of materials technology [12], in particular, the processes of molecular self-assembly, that have been applied to the development of new materials. Large structures can be easily accessed using bottom-up synthesis since they are composed of small molecules that require

fewer steps to synthesize. Therefore, most of the bottom-up approaches to nanotechnology are based on supramolecular chemistry [13] and many smart materials [14] are based on molecular recognition [15].

Within inorganic chemistry, in catalytic processes, an important application of supramolecular chemistry is the design and understanding of catalysts and catalysis. Noncovalent interactions are extremely important in catalysis, linking reactants in conformations suitable for the reaction and decreasing the reaction energy of the transition state. Encapsulation systems such as micelles, dendrimers and cavitands [16] are also used in catalysis to create suitable microenvironments for reactions (or steps between reactions).

Design based on supramolecular chemistry has led to numerous applications in the creation of functional and therapeutic biomaterials [17]. Supramolecular biomaterials provide several modular and generalizable platforms with specific mechanical, chemical and biological properties. These include systems based on supramolecular assembly of peptides, host-host macrocycles, high affinity hydrogen bonds, and metal-ligand interactions. A supramolecular approach has been used extensively to create artificial ion channels for the transport of sodium and potassium ions into and out of cells [18].

Supramolecular chemistry is also important to the development of new pharmaceutical therapies by understanding the interactions at a drug binding site. The field of drug delivery has also made critical advances as a result of supramolecular chemistry providing encapsulation and targeted release mechanisms [19]. In addition, supramolecular systems have been designed to disrupt protein-protein interactions that are important to cellular function [20].

Analytical Chemistry has also benefited from Supramolecular Chemistry, which has led to the birth of the discipline called Supramolecular Analytical Chemistry [21]. Highly attractive applications have been developed regarding the extraction of organic compounds [22,23] and metals [24] by using supramolecular solvents, hemimicelles and admicelles [25]. On the other hand, cyclodextrins and their derivatives have been used successfully as constituents of mobile or stationary phases for the separation of enantiomers in chromatographic or electrophoretic techniques [26,27]. The effects caused by solutes in certain parameters of the supramolecular aggregates (critical micelle aggregation or degree of binding) have also been exploited for the development of new

measurement parameters [28,29]. Finally, micellar catalysis has been successfully exploited for the determination of metals [30,31].

1.2 Self-assembly

Self-assembly is defined as the chemical mechanism by which two or more components are associated spontaneously and reversibly to form aggregates of larger size. All this process occurs through noncovalent interactions. Self-assembly is considered a relatively complex process which is usually divided into 3 very characteristic stages: (I) recognition between molecules, (II) sequential growth between components in a cooperative way and (III) scope of the system's fullness, which is indicated by some type of signal producing the end of the process [3].

Self-assembly is intrinsically dynamic and adaptive. Depending on the environment, the same units can yield different structures. A change in the conditions results in aggregates being reformed and indistinguishable from the original, allowing the design of tailored materials [3].

Self-assembly is responsible for the generation of numerous biological structures; for example, the double helix structure of deoxyribonucleic acids (DNA) through the recognition of purine and pyrimidine units [32]. Advances in self-assembly and the synthesis of macromolecular architectures have helped to obtain a better understanding of biological systems [33,34].

Above their critical aggregation concentration (cac), a solution of amphiphiles self-assembles [33,35]. The process is driven by a balance of attractive and repulsive forces and subtle solute-solvent and solute-solute interactions. Aggregation is a start-stop process. More aggregates of the same size are created after the addition of more molecules. Usually, solvophobicity drives aggregation while the stop process is induced by repulsion between head groups [36].

The morphology of the supramolecular aggregates depends on the relationship between the size of the polar group and the hydrophobic chain of the surfactant and can be predicted by the equation developed by Israelachvili [37].

$$g = \frac{V}{a_0 l_c}$$

being g the packing factor, V the volume of the hydrophobic chain, a_0 the average area occupied by the polar head in the aggregate, and l_c the length of the hydrophobic chain of the surfactant (Figure 1). Therefore, the parameter g depends on the number of hydrocarbon chains and carbon atoms and their degree of saturation and the size and charge of the polar head. In addition, the properties of the solution (pH, ionic strength, temperature, presence of co-surfactant) are implicitly included in V, a_0 and l_c.

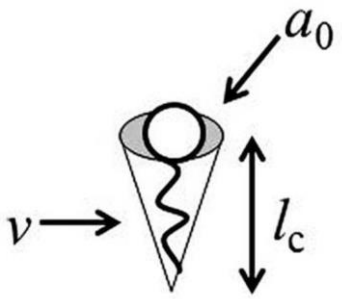
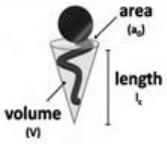
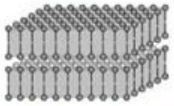


Figure 1. Parameters that determine the morphology of the supramolecular aggregate

The size, structural arrangement and complexity of the systems formed will depend also on the value of g. Among other variables, the size of the aggregates is determined fundamentally by the relationship between the size of the polar group and the length of the hydrocarbon chain. The main structures formed are shown in Table 1.

Table 1. Morphology of the supramolecular aggregates according to the parameter of packaging [35]

Type of aggregate	Packaging parameter	Amphiphile geometry	Structure of the aggregate
Spherical micelles	$\frac{V}{a_0 l_c} < \frac{1}{3}$		
Cylindrical micelles	$\frac{1}{3} < \frac{V}{a_0 l_c} > \frac{1}{2}$		
Bilayers or flexible vesicles	$\frac{1}{2} < \frac{V}{a_o l_c} < 1$		
Flat bilayers	$\frac{V}{a_o l_c} \sim 1$		
Inverse micelles	$\frac{V}{a_a l_c} > 1$		

2. Supramolecular solvents in analytical extraction processes

2.1. Synthesis of supramolecular solvents (SUPRAS)

Coacervates or supramolecular solvents are nanostructured liquids generated from colloidal solutions of amphiphiles through spontaneous phenomena of self-assembly and coacervation [38]. This new rich in colloid liquid phase is in equilibrium with the bulk solution, which contains the amphiphile at the critical aggregation concentration. The term supramolecular solvents (SUPRAS) was introduced by our research group [39] aiming to enhance the nanostructured aspects of these solvents and their very specific properties, different from those shown by ionic liquids and organic solvents. Coacervates were reported a long time ago by colloidal scientists, such as Bungenberg de Jong and Kruyt who first described coacervation [40]; by biologists like Oparin, who proposed that life first formed in coacervate droplets [41]; and by analytical chemists, when Watanabe and Tanaka introduced the cloud point (CP) technique for extraction processes [42].

The synthesis of supramolecular solvents is very simple as can be seen in the Figure 2 [43]:

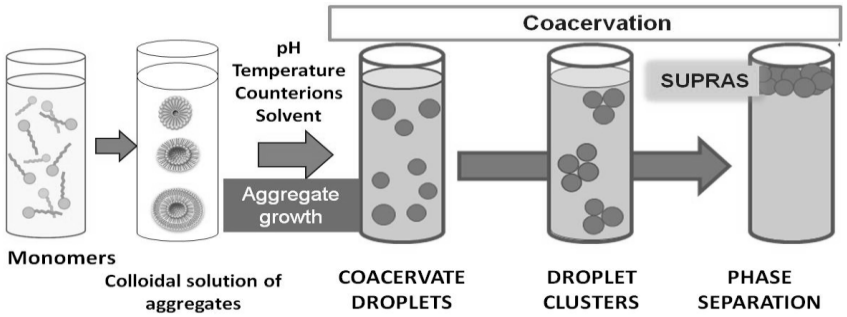


Figure 2. Scheme of the processes of self-assembly and coacervation involved in the synthesis of supramolecular solvents

A solution of monomers of amphiphiles gives three-dimensional aggregates above a critical aggregation concentration. To produce the supramolecular solvent, these aggregates must grow. This growth involves reducing the repulsion between the head groups that stops aggregation in the colloidal solution. This can be achieved by changing the environmental conditions, such as a change in the pH or the temperature of the solution, or by the addition of a salt or a poor solvent for the amphiphile. When the aggregates grow, coacervate droplets are formed [38], which associate as clusters of individual droplets and finally, these clusters are separated as a new liquid phase (coacervate or SUPRAS). These droplets hold as individual entities in the SUPRAS (Figure 3), resulting in these solvents having an intrinsically high superficial area, which facilitates extraction processes by enhancing solute mass transfer. This fact can be illustrated by photographs (Figure 4) reflecting some droplets of coacervate formed in the bulk solution (A), the formation of the two liquid phases (rich and poor in colloid) (B) and SUPRAS nanostructures (C).

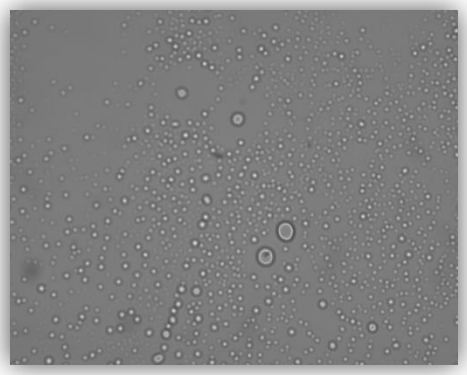


Figure 3. Micrograph of a coacervate obtained by optical microscopy

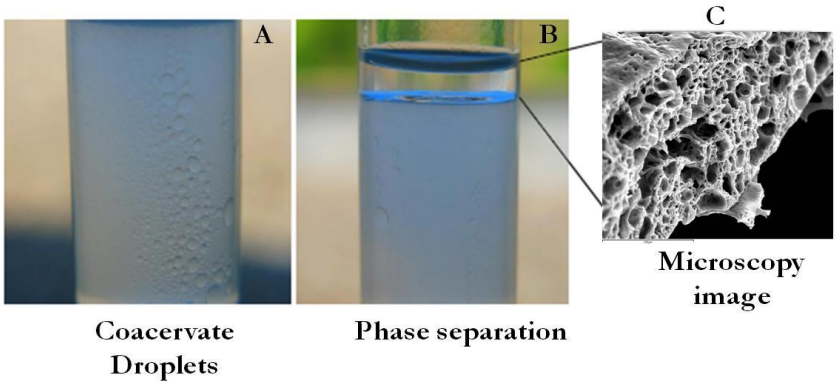


Figure 4. SUPRAS formed in ternary mixtures of 1-decanol, tetrahydrofuran and water (A) before and (B) after the centrifugation of the solution, and (C) a photograph of the supramolecular structure obtained byscanning electron microscopy

2.2. Strategies to induce coacervation

The strategy followed to induce coacervation mainly rests on the nature of the amphiphile and solvent forming the colloidal solution [22]. In principle, the energetic cost of bringing the polar heads together is much smaller for neutral amphiphiles than for ionic ones [44]. Thus, the attractive interactions between the surfactant molecules (dispersion interactions between their hydrocarbon chains) favor aggregation, while the repulsive ones disfavor it. Considering this type of phenomenon, it is obvious that nonionic surfactants present a greater facility to form aggregates due to the absence of the strong repulsive electrostatic interactions that take place between the charged groups of ionic surfactants.

Regarding nonionic amphiphiles, lowering the number of solvent molecules available for solvation is the most effective way to induce coacervation. This can be achieved by modifying the temperature in amphiphile-aqueous systems or by the

addition of a poor solvent in amphiphile-organic systems. In the case of ionic amphiphiles, coacervation must be carried out by the neutralization of the charge by adding inorganic or organic salts or amphiphilic counterions. It should be noted that ionizable amphiphiles can be considered as nonionic or ionic depending on the pH of the solution. Therefore, in addition to the already mentioned strategies, coacervation of ionizable amphiphiles can be also carried out in acid media, by the addition of an excess of the amount of acid required for their neutralization [43].

Temperature-induced coacervation is usually the proposed way to promote SUPRAS from aqueous colloidal solutions of non-ionic (e.g., alkyl and alkylphenolethoxylated), zwitterionic (e.g., alkyl betaine) and mixtures of non-ionic and non-ionic/ionic amphiphiles. The temperature at which turbidity appears and amphiphile aggregates experienced coacervation is named cloud point (CP). CP values depend on the chemical structure and concentration of the surfactant, the ionic strength of the solution and the the presence of organic compounds [45,46]. The value of CP for producing coacervation in aqueous mixtures of nonionic surfactants is the average value of the temperatures at which the surfactants coacervate individually [47]. On the other hand, CP values will be higher in mixtures of ionic and nonionic surfactants due to the fact that the repulsive forces between the charged groups of the ionic surfactant molecules disfavor aggregation [48]. With respect to amphoteric surfactants, the formation of SUPRAS will be favored when there is a decrease in the temperature of the solution below a critical value. This critical value will depend on the structure and concentration of the surfactant, the pH and the ionic strength [49].

Solvent-induced coacervation has been proved for the synthesis of SUPRAS from nonionic carboxylic acids [50] and alkanols [51]. In this approach, a poor solvent for the amphiphile is added to the colloidal solution. It should be noted that the colloidal solution solvent and the one used as coavercating agent must be miscible. Several solvents have been reported for colloidal solution (e.g., tetrahydrofuran, ethanol, dioxane, methanol, etc.) whilst water has been primarily used as the coacervating agent. The SUPRAS is produced at a relative proportion of water and organic solvent that depends on the dielectric constant of the latter and the hydrocarbon chain of the amphiphile.

On the other hand, in ionic surfactants, the reduction of the repulsive electrostatic interactions between their charged groups favors the formation of SUPRAS. There are

many processes that have been used to promote this formation, including the addition of amphiphilic or acid counterions as well as the addition of salts (NaCl and Na_2SO_4) [52,53] to reduce such electrostatic interactions. Another strategy for the formation of SUPRAS is based on the use of tretralkylammonium counterions in aqueous solutions containing equimolar mixtures of alkylcarboxylic acids (HAC) and alkylcarboxylates (AC-) [54]. The counterion, usually tetrabutylammonium (Bu_4N^+), neutralizes the negative charges of the AC- molecules on the surface of the vesicles constituted by HAC and AC- which favors the formation of large vesicles [55].

Acid-induced coacervation has been the most commonly used approach to induce SUPRAS from colloidal solutions of ionizable amphiphiles (e.g., alkyl sulphates, sulfonates and sulfoccinates) [56]. Depending on the structure formed as well as the concentration of surfactant used, the concentration of acid nedded in the coacervation process will vary [57–59]. In any case, the pH of the solution must be below the pKa of the surfactant and therefore, the acid concentrations used are very high (3-4 M) [60].

2.3. Phase Diagrams

Formation of SUPRAS occurs in a specific range of concentration of amphiphile under the action of an interval of operating conditions for the inductor agent (e.g., temperature, counterion, solvent, acid, etc.). Therefore, the first step in the synthesis of new SUPRAS is to construct the phase diagram and delimit the SUPRAS formation region [43].

Figure 5 shows different phase diagrams for typical amphiphile and coacervating agent systems. Depending on their nature, phase diagrams are more or less complex and the region for SUPRAS formation is narrower or broader [49,50,53,61,62].

The effect of the temperature in the formation of the SUPRAS will be dependent on the length of the hydrocarbon chain (increases as the number of carbons decreases) [63]. The boundaries for SUPRAS formation in phase diagrams can be influenced by the presence and concentration of additives in the colloidal solution or modifications in the operating conditions (e.g., temperature, pH, etc.). This can be exploited as a way of improving experimental extraction conditions. Thus, as the concentration and charge of inorganic ions in the colloidal solution increases (e.g., Fig. 5a), the value for CP decreases. By this approach, SUPRAS from aqueous colloidal solution of nonionic amphiphiles can be achieved at room or near-room temperature. Furthermore, this is an

aspect to should be considered when SUPRAS are applied for the extraction of solutes from real samples. The region of SUPRAS formation can be narrowed or extended by matrix components such as sugars and alcohol in foods [39]. Therefore, it is always recommended to characterize SUPRAS phase diagrams in the presence of the matrices which will be subsequently extracted.

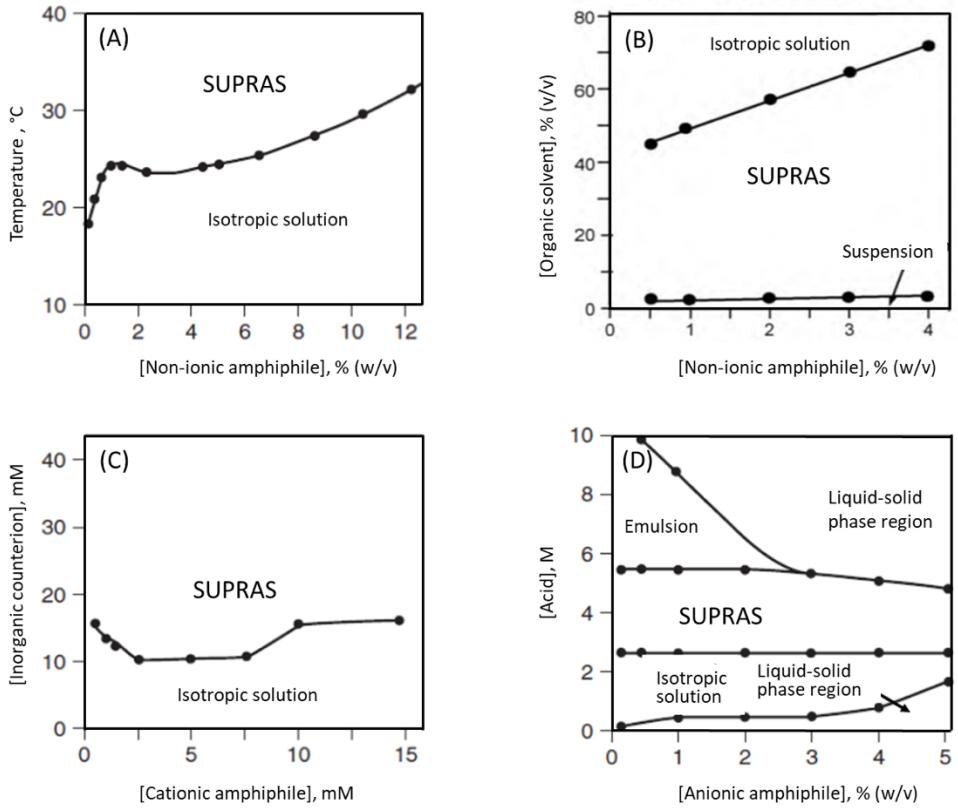


Figure 5. Phase diagrams for typical amphiphile coacervation- inducing agent systems: a) non-ionic amphiphile (temperature as coacervationinducing agent); b) non-ionic amphiphile (poor-solvent as coacervation-inducing agent); c) cationic amphiphile (inorganic counterion as coacervation inducing agent) and d) ionizable amphiphile (acid as coacervtion-inducing agent)

2.4. Solubilization of solutes in SUPRAS and concentration factors

Supramolecular solvents present regions of different polarity, viscosity and acidity. This property is responsible for the wide range of compounds, in terms of polarity, that can be solubilized into them. SUPRAS also contain a high concentration of the amphiphilic compound, generally 0.1-1 mg/μL, and therefore have the ability to solubilize a high amount of solute using small volumes of solvent.

Independently of the type of amphiphile making up the SUPRAS, the hydrocarbon region is always non-polar, making possible to predict solubilization of compounds in this region from their octanol-water constants. Forces-driving extraction of apolar compounds mainly includes dispersive, dipole-dipole, and dipole-induced dipole interactions. On the other hand, the solubility in the polar region of SUPRAS is dependent on the interactions that can be established (ionic, hydrogen bonds, polar, π-cation, etc). So far, the most frequent polar groups used in extraction processes include polyethylene oxides, carboxylic acids, sulfates, sulfonates, carboxylates, and ammonium and pyridinium ions. For polar compounds, hydrogen bonding is an extremely effective retention mechanism. Its binding energy depends on the length of the hydrocarbon chain of the amphiphile (e.g., heptanol, octanol, decanol, dodecanol between anothers).

An outstanding characteristic of SUPRAS is their ability to extract other amphiphiles by means of mixed aggregates with the amphiphiles in the ordered aggregates. Polar and hydrophobic interactions govern the formation of these mixed aggregates. Interactions get progressively stronger starting at mixtures of amphiphiles with the same polar group (e.g., non-ionic-non-ionic) up to those of opposite charge (i.e., anionic-cationic), with mixtures of nonionic-cationic and nonionic-anionic polar groups showing intermediate binding energies.

Concentration factors for solutes in SUPRAS depend on the concentration of the amphiphile and the environment for coacervation. Generally, the volume of SUPRAS depends linearly on the concentration of amphiphile used for coacervation. Therefore, top concentration factors are achieved for the lowest amphiphile's concentration. Thus, the theoretical concentration factor for solutes extracted from aqueous samples using SUPRAS of reverse dodecanoic acid micelles increases from 54 to 203 when the surfactant in the synthesis decreases from 300 to 50 mg [51].

The influence of environmental conditions on the concentration factors will depend on the coacervation agent used and should be determined for each SUPRAS. Thus, for non-ionic surfactants, an increase in the difference between the temperature used in the extraction and the critical temperature for the formation of SUPRAS causes a decrease in the volume of SUPRAS formed and an increase in the concentration of surfactant in the solvent. This same effect is observed when the acid concentration is increased, in the case of SUPRAS of anionic surfactants. For water-induced SUPRAS, the volume formed is exponentially depedent on the percentage of organic solvent in

the synthetic solution (e.g., the theoretical concentration factor increases from 38 to 94 when the percentage of THF in the synthetic solution decreases from 25 to 12%) [64].

2.5. Formats

SUPRAS has been used for extraction of solutes, by means of conventional and miniaturized techniques, in liquid and solid samples [22,43]. Regarding liquid samples, the extraction of solutes always implies the *in-situ* formation of the SUPRAS. Therefore, SUPRAS generation and solute extraction occurs in a single step. Figure 6 shows the most frequently used general scheme.

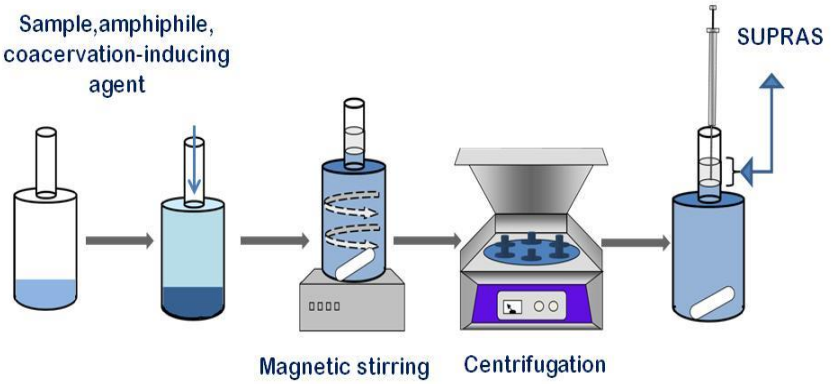


Figure 6. General scheme for the extraction of solutes in liquid samples with SUPRAS

In this format, the amphiphile (0.1-2% w/v) is added to the sample (10-100 mL) and the environmental conditions required for coacervation are established. The SUPRAS spontaneously forms in the sample, stirred to favour analyte extraction, and then, centrifuged for SUPRAS separation. The total volume of SUPRAS, or an aliquot, is then removed and analyzed directly or after dilution with organic solvent. Most non-ionic and amphoteric surfactants give rise to dense and viscous SUPRAS that must be diluted with organic solvents before their introduction into the chromatographic system. The SUPRAS of ionic surfactants, alkanols and alkylcarboxylic acids are less dense than the equilibrium solution and less viscous [50,52,56,57,65], so they can be easily extract using a microsyringe and introduced directly into the chromatographic system.

When extracting liquid samples, it is essential to know the volume of supramolecular solvent generated to determine extraction efficiency. The volume of solvent generated can be measured by picking up the SUPRAS with a graduated syringe,

but this procedure is not suitable for viscous SUPRAS. The measurement can also be carried out indirectly from the expression provided by the volume contained in a cylindrical vessel: $v = \pi r^2 h$, where r is the radius of the cylinder and h its height. In the case of SUPRAS less dense than its equilibrium solution, more accurate measurements can be made using tubes such as those shown in Figure 7, in which the diameter of the tube is reduced in its upper part, where the SUPRAS will be located, which allows to measure more accurately the height of the cylinder occupied.

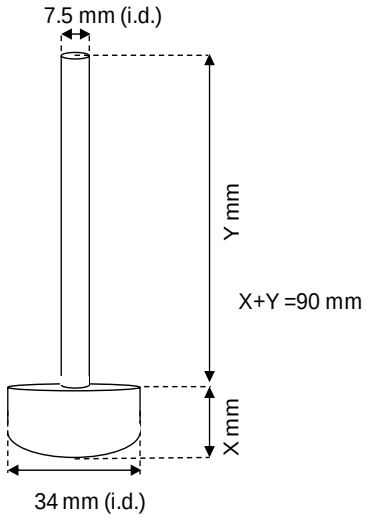


Figure 7. Scheme of a coacervation tube

SUPRAS volume can be estimated as a function of the amphiphile amount and the coacervating agent, once it has been standardized by establishing equations for volume prediction [65].

In the case of the extraction of solutes in solid samples (usually 0.1-1 g), this can be done in two different ways depending on how SUPRAS is synthesized (*in situ* or *ex situ*). Regarding *in situ* extractions, a solution containing the surfactant is added to the sample and the environmental conditions required for coacervation are established. As in liquid samples, the SUPRAS generation process and solute extraction occurs in a simple stage. After centrifugation, three phases are obtained; a solid residue consisting of the insoluble components of the matrix, an aqueous or hydroorganic equilibrium solution and the SUPRAS extract. This procedure is very suitable for the extraction of apolar compounds, since the solubilization of these occurs preferentially in the SUPRAS [66,67]. The main advantage of the *in situ* synthesis is that the the equilibrium solution

can play two different roles: humidification of the sample and trapping of polar interferences. However, since polar solutes can distribute into the SUPRAS and the equilibrium solution decreasing extraction efficiencies, its use is not recommended for the extraction of this kind of compounds.

Ex situ synthesis is more operationally convenient since a high volume of SUPRAS can be simultaneously synthesized (typically for treatment of up to 20–30 samples) and polar analytes are more efficiently extracted [68].

Supramolecular solvents have also been proposed for single-drop microextraction (SDME) [69], microextraction based on solidification of floating droplet (ME-SFD) [70] and hollow fibre-liquid phase microextraction (HFLPME) [71]. SUPRAS extend the applicability of these techniques to areas where the use of nonpolar organic solvents is less effective or not suitable, e.g., when the separation of the analytes is carried out by LC or when the analytes are polar or ionic compounds. SUPRAS consisting of vesicles of alkylcarboxylic acids are especially compatible for SDME, ME-SFD and HFLPME because of their poor solubility in water and their capability to arrange as spherical droplets. These properties arise from the high cohesiveness and stability shown by these SUPRAS thank to the strong hydrogen bonding interactions established between the head groups of the amphiphile molecules. SDME, ME-SFD and HFLPME in combination with vesicular SUPRAS have been used for the extraction of chlorophenols [69], parabens [70] and halogenated anilines [71] in aqueous samples.

Figure 8 shows, as an example, the system used for SUPRAS-based SDME. The formation of spherical and stable drops of SUPRAS at the tip of conventional microsyringes depends on the type of intermolecular forces that are established between the polar heads of the surfactant. Hydrogen bridges are sufficiently stable to allow the formation of spherical droplets, so SUPRAS composed of carboxylic acids are ideal for this application.

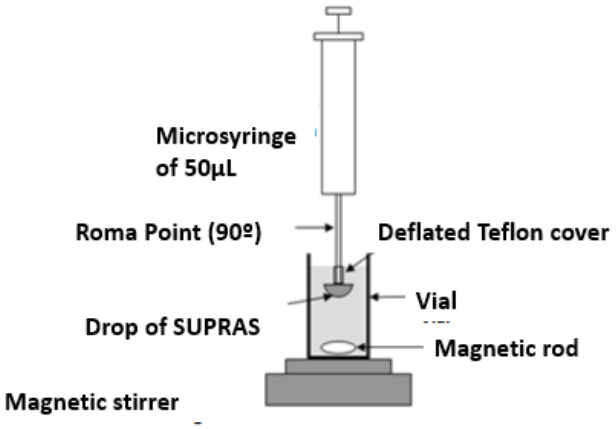


Figure 8. Diagram of the supramolecular microextraction process with a drop

In addition to miniaturization, another trend in extraction with SUPRAS is automation. The first advances in this direction were made by Fang et al. [72] by using a SUPRAS of a nonionic surfactant in flow injection analysis (FIA). The greatest difficulty in the use of this extraction format is the formation of the SUPRAS as well as phase separation. This problem was solved by the addition of a salt instead of the use of temperature for phase separation, the elution being carried out with an organic solvent. Online couplings have also been developed for liquid chromatography coupled to fluorescence detection (LC-Fl) [73]. For this approach, the aqueous samples, to which the surfactant has been added (Tergitol 15-S-7), are injected into a six-way LC valve and ammonium sulfate is added to induce phase separation. The phase rich in surfactant and analytes (PAHs) is retained on a column of silica gel. After a few minutes the valve is changed to the injection position, so thus the mobile phase carries the analyte and the surfactant to the LC-Fl system.

2.6. SUPRAS compatibility with separation/detection systems

The use of SUPRAS in combination with different separation/detection techniques has been very extensive and continues to be so in recent years. Supramolecular solvents have been used mainly in combination with liquid chromatography (LC) coupled to UV-visible detectors, fluorescence and mass spectrometry (MS). After the analytical extraction with the SUPRAS, they are injected directly into the chromatograph without a previous dilution or clean-up stage, because

SUPRAS are usually destroyed in the hydro-organic mobile phase, generating a high concentration of surfactant that can be seen at a single chromatographic peak. Occasionally, when a high percentage of water is used (> 40%), surfactant aggregates are not destroyed, creating a pseudo-phase where analytes may partition. As a result, retention times for analytes may vary and chromatographic resolution could be affected. That is why, in this case, a dilution stage of the extract in organic solvent prior to its chromatographic analysis is advised.

The physicochemical properties and structure of the surfactants used are very important to know because, depending on them, a greater chromatographic separation and better analytical detection can be achieved. An example of the importance of a good choice are non-ionic surfactants. For example, those belonging to the series of Triton X (octyl phenols ethoxylates) and PONPE (nonyl phenols ethoxylates) are only available as a mixture of homologs in the market. Therefore, they are not a good option for the separation/detection of compounds of medium/high polarity, since very wide chromatographic peaks occur that coelute with the solutes of interest. It is interesting to mention that the presence of aromatic rings in the structure of some surfactants produces high signals due to their high absorbance. This disadvantage has been solved by the use of ionic or zwitterionic non-aromatic surfactants because they elute at low retention times, producing narrow chromatographic peaks whose absorbance is below 210 nm.

When using mass spectrometry, the first thing to considere is that the surfactant used must have a different retention time than the analytes of interest. That is why, as a general rule, the window of elution of surfactant after the chromatographic separation is sent to waste. In this way, the contamination of the source and the possible loss of efficiency in the ionization of the solutes, which could produce a decrease in the sensitivity, are avoided.

In the last decade, important advances have been made in the coupling of extractions based on SUPRAS and analysis by gas chromatography (GC). For this purpose, strategies have been developed focused mainly on the elimination of the surfactant (normally Triton X-114) before the injection. Thus, SPE columns have been used to retain the surfactant and elute the analytes (e.g., silica gel and Fluorisil columns in series for pesticides [74]) or vice versa, with ion exchange columns in the case of charged analytes, as for example phenothiazines [75]. The re-extraction of the analytes

into an immiscible solvent with water assisted by microwaves or ultrasound has also been proposed for PAHs [76], pesticides [77] and alkaloids [78]. In recent years, derivatization of the surfactant prior to its introduction into the chromatographic system has also been proposed. The Triton X-114 surfactant, derivatized with N, O-bis (trimethylsilyl) trifluoroacetamide, is transformed into a volatile compound that provides a narrow peak that does not interfere in the chromatographic determination of PAHs, herbicides and profens [79].

SUPRAS are also compatible with some modes of capillary electrophoresis (CE). However, the coupling of SUPRAS with CE has not been a fruitful area to date. Although the use of surfactants in capillary electrophoresis (CE) is a common practice, either as additives or as components of micelles in micellar electrokinetic chromatography (MEKC), the use of SUPRAS for extraction and subsequent CE analysis is not very widespread. Some progress has been made in the combination of SUPRAS with the mobile phases used in capillary electrochromatography (CEC), since this technique is a hybrid between CE and LC. The only post-extraction treatment that is required in this type of CE is the dilution of the extract with an organic solvent to avoid capillary obstruction. This strategy has been applied to the determination of PAHs, polychlorinated dibenzo-p-dioxins (PCDDs) and phthalates [80,81] using a C18 stationary phase. On the other hand, satisfactory results have been obtained by combining extractions with SUPRAS and capillary zone electrophoresis (capillary zone electrophoresis, CZE) through the use of non-aqueous media to avoid adsorption of the surfactant on the capillary wall [82,83]. This adsorption causes a high loss of both efficiency and reproducibility with respect to retention times, as well as electrophoretic peaks [84]. It has also been possible to combine SUPRAS extracts diluted with organic solvent and MEKC, usually methanol, with dilution factors of 1.5-3 to reduce its viscosity. In this way, phenolic compounds in water [85] or malachite green in fish have been successfully analyzed [86]. Finally, strategies have also been addressed to eliminate the surfactant before its introduction in CE. Thus, the re-extraction of the analytes in aqueous solution has been carried out prior to derivatization to confer them hydrophilic character [87], based on their acid-base characteristics [88]. With this approach, extracts are obtained practically free of surfactant, thus avoiding their interference in the injection and electrophoretic separation. This methodology has been applied to the determination of phenol, m-nitrophenol and auxins [87,88].

2.7. Applications

SUPRAS have found extensive applications in the analytical extraction of metals and organic compounds from environmental, food, and biological samples thank to their remarkable and efficient solubilization of solutes in a wide polarity range [22]. They are especially suitable for multiresidue analysis. Several reviews identified and described the major features and analytical characteristics of SUPRAS-based extractions [23,24,44,60,89–96].

Temperature-induced SUPRAS, formed by polyethoxylated non-ionic surfactants, have been by far the most used in extraction processes. One of the main reasons for this high use is the scarce attention that has been paid to the development of alternative SUPRAS by the researchers involved in this area. Among all, we can highlight the polyoxyethylated nonionic surfactants whose concentration most commonly used is in the interval 0.1-2%, although quantitative extraction of bioactive compounds in biological samples often requires much higher concentrations (3-10%) [22]. This percentage leads to low concentration factors. As a consequence, the detection limits obtained (in the order of $\mu g\ L^{-1}$) are not sufficiently low to determine organic pollutants at the concentrations that are often found in environmental aqueous samples (in the order of $ng\ L^{-1}$). The applications developed have focused mainly on the extraction of PAHs [97–99], pesticides [77,100–103] and bioactive compounds [77,104,105] present in environmental, food and biological samples, respectively. Interesting applications have also been developed related to the extraction of dyes [106,107], endocrine disruptors [108] and phenols [109]. The majority of the extractions are quantitative (the recovery percentage is usually in the 80-100% range). Although there are many non-ionic surfactants that are commercially available, Triton X-114 continues to be preferred for the formation of this type of SUPRAS, followed by Triton X-100 and Genapol X-080.

The use of mixtures of ionic and nonionic surfactants is an excellent strategy for the extraction of ionic compounds. The most common coacervating agent is temperature (40-85 ºC), in the presence or absence of salts. As has been observed in the majority of the analytical applications described, Triton X-114 is the non-ionic surfactant most commonly used in these mixtures, together with cetyltrimethyl ammonium bromide and sodium dodecyl sulfate, for the extraction of anionic and cationic compounds, respectively. This type of SUPRAS has been successfully applied

to the extraction of a large variety of analytes such as pesticides, dyes and humic and fulvic acids in environmental and biological samples [110]. Regarding the mix of two non-ionic surfactants, they have been proposed for applications such as the extraction of PAHs. This is because, apparently, these SUPRAS have a higher active area with respect to the studies carried out with simple non-ionic surfactants. In addition, they provide greater selectivity. The greatest disadvantage of these micelles is the high necessary working temperature (78ºC) [110,111]. In the same way as for the SUPRAS formed from aqueous nonionic micelles, UV detection has been the preferred detection system after the chromatographic separation of the extracts, obtaining limits of detection at levels of $\mu g\ L^{-1}$.

Water-induced SUPRAS, made up of carboxylic acids or alkanols, have marked a turning point in this area. One of the most valuable assets of this type of supramolecular extractants is the high concentration of amphiphiles they contain (> 0.75 mg/μL). Therefore, this important property allows to obtain large concentration factors in liquid samples, e.g., 569 for bisphenols and their corresponding diglycidyl ethers in environmental waters, with typical SUPRAS/solid sample ratios of 1/1 [112]. Due to the ability of these SUPRAS to establish hydrogen bonds, as well as polar interactions with solutes, the extraction of polar compounds is really effective. The applications of SUPRAS formed from reverse micelles have focused on the extraction of a wide variety of organic compounds such as PAHs, dyes, endocrine disruptors, mycotoxins or bioactive substances, in environmental and agro-food samples [68,113–115].

Regarding acid-based SUPRAS, the strong experimental conditions required for coacervation (e.g., 3-4 M HCl) is their main handicap for their routine application. However, such acidic conditions have been recognized as essential when proccessing very complex environmental solid samples (e.g., soil, sludge, and sediments). For these matrices, the acid medium was shown to favor desorption of cationic surfactants from the sludge, probably because of an ion-exchange mechanism [116]. These SUPRAS allow high concentration factors to be reached at low concentrations of surfactant (eg. 140 with 0.1% dodecanesulfonate [117]). The main advantage with respect to non-ionic surfactants is their commercial availability, as well as low retention times in LC. These two qualities make them extremely valid for applications in mass spectrometry because the chromatographic peaks are narrow and can thus be easily sent to waste after the chromatographic separation.

Coacervation of counterion-induced SUPRAS commonly requires the addition of inorganic salts at high concentrations (e.g., 400 g/L of NaCl for cetrimide) and the presence of a cosurfactant, restricting their applicability. On the other hand, for SUPRAS induced by amphiphilic counterions, the conditions are mild. The coacervation of vesicular mixtures of carboxylic acid-carboxylates in the presence of tetrabutylammonium salts is a good example. These SUPRAS contain the higher concentration of amphiphiles, presenting a concentration in the order of 1 mg/μL. This large concentration of amphiphiles has allowed them to present the largest values to what preconcentration factors are concerned (e.g., between 18 and 1334 for decanoic concentrations from 4% to 0.025%, respectively). Among other properties, they also present advantages such as, for example, the different types of interactions they can establish: ionic, hydrogen bonding, π-cation and hydrophobic bridges, the great cohesion forces between the molecules that form the aggregates, and their high kinetic stability. Among the many applications described to date for these SUPRAS, it is worth highlighting its great success in the extraction of pesticides, endocrine disruptors and phenols in food and environmental samples [118–120].

3. Tailored supramolecular solvents

SUPRAS are usually formed by amphiphiles, water, and, where appropriate, a coacervating agent and/or other additives. The concentration of amphiphiles in the SUPRAS can be tailored by the proper selection of the amphiphile and/or the environment for coacervation [22]. SUPRAS tailoring is a consequence of its adjustability since the forces that drive self-assembly and coacervation of amphiphiles are always noncovalent and, as a result, the synthesis process is reversible. The aggregates that form the SUPRAS are obtained by means of a balance between the attractive forces generated by the hydrocarbon chains and repulsion obtained from the polar groups. If the environmental conditions reduce or eliminate these repulsive forces, the growth of the aggregates and therefore the coacervation will be favored. In the opposite case, in which the repulsive forces are favored by said environmental conditions, the coacervate will be destroyed forming aggregates of a smaller size, that is, the monomers form the first colloidal solution. This means that there is an opportunity to adjust the composition, structure, size and properties of the SUPRAS according to

the previously set requirements and, therefore, custom solvents with specific functions (tailored SUPRAS) can be synthetized.

3.1 Tailoring SUPRAS composition

Usually, as the length of the hydrocarbon chain of the amphiphile increases, the concentration of amphiphile in the SUPRAS gradually decreases (e.g. for alcohol ethoxylates: 1.56, 1.35, 0.45 and 0.34 M for $C_{10}E_6$, $C_{12}E_6$, $C_{14}E_6$ and $C_{16}E_6$, respectively). A Similar behavior has been reported for SUPRAS formed by amphiphiles containing ethoxylates or oxyethylates head groups (e.g., for alcohol ethoxylates: 2.11, 1.06, and 0.84 M for $C_{12}E_5$, $C_{12}E_7$ and $C_{12}E_8$, respectively). Special attention deserves the high concentration of amphiphiles in SUPRAS made up of carboxylic acids and alkanols, offering a high number of binding sites for solute solubilization (e.g. 3.66, 3.19 and 3.17 M for octanoic, decanoic and dodecanoic acids, respectively) [22].

SUPRAS composition can also be tailored by modifying the coacervation environment. For example, the concentration of amphiphile in SUPRAS depends on the coacervation temperature and the presence of some additives in the colloidal solution (e.g., electrolytes and organic compounds). The increase induced by temperature can be rationalized buy considering the breaking of hydrogen bonds between the oxyethylated groups and water and, as a result, the reduction of the content of water in the SUPRAS.

The influence of electrolytes, in the case of ethoxylated SUPRAS, is highly dependent on the type of anion and cation and their respective concentrations. Because of their salting-out effect, the concentration of amphiphile increases in the presence of cations such as Na^+, K^+, Rb^+, Cs^+, and NH_4^+ and most of anions. However, most cations and some anions such as SCN^- and I^- cause the salting-in effect and, subsequently, a decrease in the concentration of amphiphile in the SUPRAS.

The composition is highly dependent on the relative proportion of water and organic solvent in the colloidal solution for water-induced SUPRAS. As the percentage of organic solvent in the colloidal solution increases, the concentration of amphiphile gradually decreases, for SUPRAS formed by carboxylic acids and alkanols. There are predictable dependences between the concentration of amphiphile in the SUPRAS and the percentage of organic solvent, allowing the prediction of the volume of SUPRAS

and, subsequently, the prediction of the theoretically achievable concentration factors. Table 2 shows, as a function of the amount of amphiphile and the percentage of THF, some of the general equations obtained for predicting the volume of SUPRAS obtained from alkanols and carboxylic acids. It should be noted that there is a lineal dependence on the amphiphile, indicating that the concentration of amphiphile in the SUPRAS has no effect on this parameter. On the other hand, there is an exponential dependence on the percentage of THF, pointing out that, as this percentage increases, more diluted SUPRAS will be obtained. Therefore, the highest concentration of amphiphile in the SUPRAS should be obtained at the lowest percentages of THF.

The concentration of amphiphile in the SUPRAS can be tailored by proper selection of the concentration of the coacervating agent for acid-induced and counterion-induced SUPRAS. Thus, as the concentration of HCl (i.e., the coacervating agent) in the synthetic solution increases, the concentration of alkyl sulfates, sulfonates, and sulfosuccinates in the SUPRAS also increases. A similar behavior is obtained for NaCl-induced SUPRAS obtained from cetrimide.

Table 2. General equations for the prediction of the volume of SUPRAS obtained from alkanols and carboxylic acids in tetrahydrofuran-water mixtures

Amphiphile	n	General equation
Alkanols	6-13	$V_{SUPRAS} = X\ (0.17 + e^{0.0389\ THF})$
$CH_3(CH_2)_nOH$		
Carboxylic acids		
$CH_3(CH_2)_n\ COOH$		
Hexanoic acid	4	$V_{SUPRAS} = 0.60\ X + 0.076\ X\ THF + e^{0.104\ THF}$
Octanoic acid	6	$V_{SUPRAS} = 1.17X\ e^{0.039\ THF}$
Decanoic acid	8	$V_{SUPRAS} = 1.05X\ e^{0.047\ THF}$
Dodecanoic acid	10	$V_{SUPRAS} = 0.92X\ e^{0.056\ THF}$

V_{SUPRAS}: Volume of supramolecular solvent (µL); X: Amount of surfactant (mg); THF: Percent THF concentration (v/v)

3.2 Tailoring SUPRAS nanostructures

SUPRAS nanostructures can also be tailored by the proper selection of the amphiphile and the environmental conditions. However, as a result of the character of the supramolecular assemblies, set by their non-covalent bonds, more progress in this area is needed [121]. Supramolecular assemblies depend mainly on the amphiphile structure, as has been demonstrated by different techniques for temperature-induced SUPRAS. The nanostructures in the SUPRAS depend on the n-to-m ratio for oxyethylated nonionic surfactants (C_nE_m). For medium n-to-m ratio, such as $C_{12}E_5$, an increase in the temperature produces a transition in the micelle from spherical to rod-like.

The amphiphiles usually arrange as cylindrical micelles, similar to $C_{12}E_5$, in temperature-induced SUPRAS formed by ionic amphiphiles in the presence of salts (e.g., erucylbis (hydroxyethyl) methylammonium chloride in the presence of sodium tosylate or salicylate, hexadecyltetramethylammonium bromide in the presence of sodium salts, and carboxylates (C14-18) in the presence of guanidine hydrochloride). Micelles are bound giving rise to a network, since the counterion is of amphiphilic nature (e.g., SUPRAS obtained from dodecyl sulfate and tetrabutylammonium).

A clear example of the influence of the environment in the supramolecular assemblies is the case of water-induced SUPRAS. Carboxylic acids and alkanols arrange, in THF-water, as inverted hexagonal aggregates (Fig. 9). Different structural parameters can be tailored just by controlling the enviroment for amphiphile self-assembly (specifically, the THF:water ratio in the bulk solution) such as the global composition of the solvent, the size of the coacervate droplets and the aqueous cavities of the inverted hexagonal. Interestingly, all previous features can all be reversed by modifying the environment, which shows that these SUPRAS are highly adaptive. As the proportion of THF increases, the size of coacervate droplets also increases (e.g., droplet sizes within the intervals around 1-5, 40-60, and 100-200 μm have been reported for decanol-based SUPRAS generated in environments containing 10% (v/v), 40% (v/v), and 60% (v/v) of THF, respectively). The hexagonal holes were assumed to be openings of the water channels from the inside of the matrix, with apparent diameters ranging from approximately a few 10ths of a micrometer to about 0.5 μm. THF was assumed to be the continuous phase dispersing the coacervate droplets forming the SUPRAS. These SUPRAS has been exploited as restricted access solvents (RAM-SUPRAS) since the size

of the vacuoles is also dependent on the percentage of THF in the colloidal solution. Interesting applications have been proved for the extraction of low molecular weight solutes while excluding macromolecules by both physical, by a size exclusion mechanism based on macromolecules not reaching the aqueous cavity, and chemical, i.e. precipitation of proteins, mechanisms.

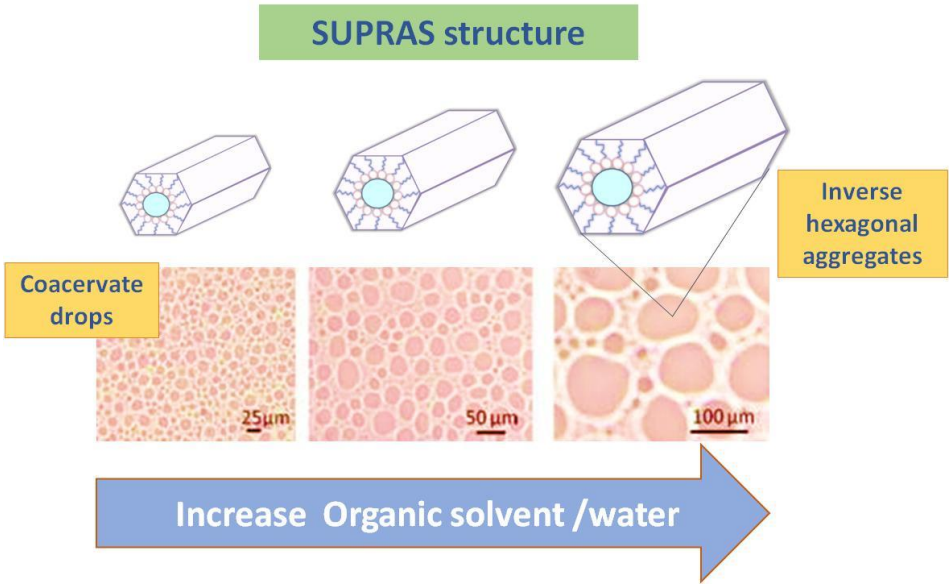


Figure 9. Organic solvent/water effect on the structure of alkanol-based RAM-SUPRAS

These properties have made them extremely valid for numerous applications using the SUPRAS as a cleaning step. Some investigations aimed to develop generalized sample treatments for food matrices with a wide range of compositions (for example, percentage of proteins, fats, carbohydrates, water, etc.) or considered complicated (for example, spices with high pigment content, composed of essential oils, carbohydrates and/or fats). An example is the determination of ochratoxin A (OTA) in wines and spices and aflatoxin B1 (AFB1) in cereals [113]. The recoveries for enriched samples were between 84% and 96%. The treatment of the sample does not require special equipment, it is robust, and the synthesis of the solvent is spontaneous and accessible to every lab. Other studies focused exclusively on solid samples [114]. It has been possible to determine that the restricted access properties of the SUPRAS allow the simplification of the treatments, reducing cleaning stages as well as increasing the selectivity when working with analytes of a wide range of polarities, including highly polar ones. An example of the application of RAM-SUPRAS to simplify sample

treatment in analytical methods is the determination of endocrine disruptors in sediments [122]. Endocrine disruptors contain hydroxyl and ketonic groups and therefore their extraction is carried out by mixed mechanisms: interaction by hydrogen bonds and Van der Waals forces. The molecular weight of these is less than 300 and the recoveries obtained with the RAM-SUPRAS are quantitative for THF percentages between 20 and 60%. The main organic components of the matrix (humic and fulvic acids) are not extracted and the sample treatment consists of a single stage; microextraction with 400 μL of SUPRAS for 10 min and direct injection of the extract into the LC-MS/MS for quantification.

4. Nanostructured lipid carriers

The oral route is the most important and conventional method for drug administration. However, oral drug delivery systems have limitations, such as drug degradation in the gastrointestinal track (by enzymes, pH…), pre-systemic metabolism or toxic side effects [123]. A novel alternative to overcome this problem is the nanoencapsulation of drugs in nanocarriers. Nanoencapsulation of therapeutic agents increases their efficacy, specificity and targeting ability. Nanocarriers protect their payload from premature degradation in the biological environment, enhance bioavailability, and prolong their presence in blood and cellular uptake [124].

Among the several core materials available for nanoencapsulation, lipids are an excellent option. Due to their favorable physicochemical properties and good permeation through the gastrointestinal barrier, lipids have been used extensively for the development of drug delivery nanocarriers and to improve the oral bioavailability issue of several drugs [125].

The traditional model of lipid carriers are liposomes, which were discovered in 1960 [126]. Since these formulations have many disadvantages, including short shelf life, poor stability, low encapsulation efficiency and cell interaction, in the years that followed, new lipid-based forms were developed [123]. In 1990, two scientists, Prof. R.H. Müller and Prof. M. Gasco, proposed a new kind of lipid nanocarriers dubbed solid lipid nanoparticles (SLNs). Their research showed that SLNs can be an alternative to organic nanoparticles (e.g. PLGA nanoparticles) and traditional lipid-based formulations (e.g. emulsions, liposomes) [123,127].

A newer generation of lipid nanoformulations, developed in 1999, is called nanostructured lipid carriers (NLCs), their first use being related to retinol [123]. NLCs are drug-delivery systems composed of solid and liquid lipids as a core matrix. It has been shown that NLCs display some advantages for drug therapy over conventional carriers, including increased solubility, the ability to enhance storage stability, improved permeability and bioavailability, reduced adverse effects, prolonged half-life, and tissue-targeted delivery. Further studies have confirmed the applicability of NLCs as carriers for many small molecules. Furthermore, drugs can be encapsulated within an NLC and be suitable for different routes of administration: oral, intravenous, pulmonary and ocular [123].

The production process is identical for SLN and NLC particles. The solid lipid or lipid blend is melted and the pharmaceutical (or active principle) is dissolved in the melted lipid phase, which is subsequently dispersed by high speed stirring in a hot aqueous surfactant/stabilizer solution of equivalent temperature. The obtained pre-emulsion is then homogenized. After cooling the emulsion, droplets crystallize forming lipid nanoparticles with a solid particle matrix that, depending on the starting formulation, may be either SLNs or NLCs. The main advantages of the later are their increased loading capacity while keeping activities compared to those shown by SLNs, and a firmer inclusion of the active principle inside the particle matrix [127].

Lipid-based delivery systems are regarded as safe and efficient and they are proving to be an attractive delivery strategy for pharmaceutical substances. However, the development of these delivery systems requires comprehensive understanding of physicochemical characteristics of drugs and delivery carriers, and of formulation and process variables [128].

4.1 NLC Components

NLCs are mainly oil in water (O/W) emulsions in which the major components are lipids, surfactants and water. Furthermore, a proportion of oil is replaced by a solid lipid resulting in a solid lipid matrix at room temperature. Solid lipids are blended with oils, preferably in a ratio ranging from 70:30 to 99.9:0.1 (%, w/w) and the emulsion is stabilized by a surfactant solution (surfactant proportion from 0.5 to 5%). Several lipids, oils and surfactants commonly used in the formulation of NLCs are listed in Table 3 [129].

4.2 NLCs methods of preparation: high pressure homogenization (HPH)

The most popular method of NLC production is high pressure homogenization (HPH), which is divided into hot and cold techniques. This method is especially common for large scale production of nanocarriers and has many advantages, such as an easy scale up and a short production time. In addition, it does not make use of organic solvents. In the hot method, a hot surfactant solution is added to a mixture of melted lipids (being melted around 5-10°C above their melting points) containing the active principle, using a high-speed stirring. The obtained emulsion is homogenized under high pressure (generally at 500 or 800 bar) through a very high shear stress, resulting in disruption of particles down to the sub- micro- or nano-meter range, creating a hot nanoemulsion. Finally, this nanoemulsion is cooled to room temperature, to obtain NLCs. In cold homogenization, the melted lipid containing the active substances is cooled down (using liquid nitrogen or ice). Then, the obtained solid is crushed and ground. The microparticles obtained are dispersed in a cold surfactant solution and then passed through a high-pressure homogenizer at room temperature [123].

4.2.1 Microemulsion technique

In the microemulsion technique, the lipids are melted, and the active principle is then incorporated. A mixture of water, surfactant and co-surfactant(s) is heated to the same temperature as the lipids and added under mild stirring to the lipid melt. This microemulsion is then dispersed in a cold aqueous medium under mild mechanical mixing in a ratio in the range 1:25–1:50. This dispersion in cold aqueous medium leads to rapid re-crystallization of the oil droplets and formation of NLCs [130].

4.2.2 Solvent emulsification-evaporation technique

For this method of preparation, the lipophilic material and the active principle are dissolved in a water immiscible organic solvent and emulsified in an aqueous phase using a high-speed homogenizer. The efficiency of this fine emulsification is improved by immediately passing the coarse emulsion through a microfluidizer. Finally, the organic solvent is evaporated by mechanical stirring at room temperature and reduced pressure leaving NLCs [130].

Table 3. Formulations commonly applied for the synthesis of NLCs

Formulation components	Examples
Solid lipid	Stearic acid
	Glyceryl Monostearate
	Carnauba wax
	Cetyl palmitate
	Glyceryl Palmitostearate (Precirol® ATO 5)
	Glyceryl Behenate (Compritol® 888 ATO)
	Witepsol®
	Softisan®
	Gelucire®
Liquid lipid	Soybean oil
	Medium chain triglycerides (MCT)/caprylic and capric triglycerides
	Oleic Acid (OA)
	Isopropyl Myristate
	α-tocopherol/ Vitamin E
	Corn oil
	Squalene
Surfactant	Poloxamer 188
	Tween® 80
	Tween® 20
	Myverol™ 18-04 K
	Sodium dodecyl sulfate (SDS)
	Sodium deoxy cholate (SDC)
	Polyvinyl alcohol (PVA)
	Lecithin
	Solutol® HS 15
	Polyoxyl castor oil

Source: Khosa et al. (2018) [129]

4.2.3 Solvent emulsification-diffusion technique

This method is based on the use of an organic solvent (oil phase) that is partially miscible with water. An oil/water emulsion is obtained upon injection of the surfactant, usually containing a stabilizing agent, under mechanical stirring followed by high-pressure homogenization. The emulsion is then diluted with a large amount of water to overcome the aqueous miscibility of the organic solvent. Due to the spontaneous diffusion of the solvents, an interfacial turbulence is created between the two phases, leading to the formation of small particles [130].

4.2.4 Melting dispersion method

In the melting dispersion method, the active principle and the solid lipid are melted in an organic solvent regarded as oil phase and, simultaneously, a water phase is also heated to the same temperature as the oil phase. Subsequently, the oil phase is added to a small volume of water phase and the resulting emulsion is stirred at high speed for a few hours. Finally, it is cooled down to room temperature to yield NLCs [130].

4.2.5 Film ultrasonication dispersion technique

NLCs can also be prepared by sonication. In this method, a thin film of lipid phase formed upon solvent evaporation is ultrasonic dispersed in the presence of an aqueous surfactant solution at elevated temperature. The subsequent cooling of the system lead to the formation of NLCs [131].

4.2.6 Solvent injection

For this method of preparation, lipids are dissolved in a water-miscible solvent or water-miscible solvent mixture and quickly injected into an aqueous solution of surfactants through an injection needle [130].

4.2.7 Double emulsion technique

In the double emulsion technique, the active principle (mainly hydrophilic drugs) is dissolved in an aqueous solution and emulsified into the melted lipid. The primary emulsion is stabilized by adding an aqueous phase containing hydrophilic emulsifiers, which is followed by stirring and filtration [130].

The main advantages and disadvantages of all these methods of preparation for NLCs are summarized in table 4.

Table 4. Methodologies for NLCs synthesis

Method	Advantages	Disadvantages
High pressure homogenization	Simple and cost effective	Increase in temperature during homogenization cannot be avoided.
a. Hot homogenization	Well established on large scale	Dispersion quality is often compromised by the presence of micro particles
b. Cold homogenization	Avoidance of organic solvent in manufacture. Thermolabile drug can be processed by cold method.	-
Microemulsion	Scale up feasible	Uses high concentration of surfactants Strong dilution of particles due to use of high volumes of water.
Solvent diffusion method	Water immiscible solvents used	Use of Organic solvents
Solvent emulsification-evaporation method	Avoids any thermal stress hence suitable for thermosensitive drugs	-
Emulsification sonication method	High shear mixing.	Additional step of evaporation required Possible metallic contamination from the probe during sonication
Phase inversion technique	Suitable for thermosensitive drugs Avoids use of organic solvents	Tedious process
Solvent injection/ solvent Displacement method	Easy handling and fast production process	Use of Organic solvents
Membrane contractor technique	Simple methodology and equipment	-

Source: Source: Khosa et al. (2018) [129]

4.3 Types of NLCs

Depending on the method of preparation and composition, NLCs can be classified as imperfect, amorphous and multiple (Figure 10).

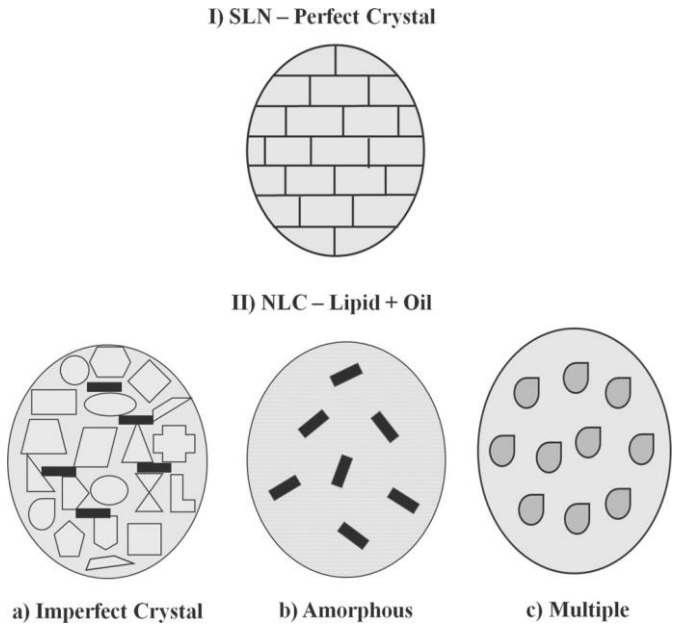


Figure. 10. Types of NLCs (II) in comparison with the ordered SLNs (I). The NLC types are: a) imperfect, b) amorphous and c) multiple [129]

The first type is an imperfectly structured solid matrix, where different lipids are mixed spatially creating imperfections in the crystal order. The second type, known as "amorphous type" has an amorphous structure, formed by mixing solid lipids with special lipids like hydroxyoctacosenyl hydroxystearate, isopropyl myristate or medium chain triglycerides such as miglyol 812. The third type is characterized for containing numerous nanosized liquid oil compartments distributed in a solid lipid matrix. In these nanocompartments the solubility of the drug is greater and, therefore, the drug load increases. [130].

4.4 Effect of components and process parameters in NLCs synthesis

Several factors, including composition of the formulation and the manufacturing process, can affect the different properties shown by NLCs. For example, a high surfactant/lipid ratio generates smaller particles and high drug concentrations lead to larger particle sizes compared to low concentrations [131].

The quality and efficiency of NLCs is influenced by the type and concentration of the surfactant and of the lipid involved in their formation. For example, an ionic surfactant having low emulsification efficiency can be used to increase the nanoparticle load, which is related to the enhancement of the electrostatic repulsion which leads to

an improved physical stability of the colloidal system. In the same way, a non-ionic emulsifier, such as Poloxamer 188, can offer an additional steric stabilization effect that prevents the aggregation of fine particles in the colloidal system [132,133].

4.5 Characterization of NLCs

Like for other colloidal carriers, characterization of NLCs is a critical requirement for assessing quality, stability and release kinetics of the delivery system. Common characterization methods are the measurement of particle size and its distribution, structural properties, surface charge and morphology of the particles, changes in crystallinity, polymorphism, and thermal behavior of the lipids [129]. The main characterization techniques are discussed below.

4.5.1 Particle size and distribution

Particle size and distribution are important characteristics that influence the stability, solubility, release rate and biological performance of NLCs. The ideal diameter of NLCs is in the range of 50-300 nm for those more usual applications (e.g., chemotherapeutic agents). In addition, the particle size is an indicator of the stability of the NLCs and should maintain a narrow range during storage since an increase in particle size indicates agglomeration and, therefore, physical instability. Different systems, such as photon correlation spectroscopy, dynamic light scattering and quasi-light scattering, are commonly used for collecting information about size, based on the equivalent hydrodynamic diameter in liquid dispersions. [129].

4.5.2 Zeta potential

Zeta potential can be defined as the electric potential in the interfacial double layer at the location of the slipping plane relative to a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. It is closely related to the morphology of the particle surface and the stability of the suspension. Unlike particle size or molecular weight, zeta potential not only depends on the particles but also on their environment (e.g., pH, ionic strength) [134]. Zeta potential provides important information about the long-term stability of nanoparticles and their

tendency to agglomerate. In general, a minimum zeta potential of ± 20 mV is desirable to exhibit a high structural stability since a high negative potential or positive potential will result in the repulsion of the particles, decreasing the tendency to aggregate. The most common methodology for measuring the zeta potential is Dynamic Light Scattering (DLS), being this function usually integrated into the particle size measurement process [131,135,136].

4.5.3 Particle morphology

Another parameter for the characterization of nanoparticles is their morphology. Morphology refers to external features, such as the shape and surface structure of the particles. Electron microscopy techniques such as scanning electron microscopy (SEM) and atomic force microscopy (AFM) are extremely useful for determining particle size, state of aggregation, morphology, surface topography and even the internal structure of the NLCs [137].

4.5.4 Crystallinity and lipid modification

Characterization of crystallinity and the degree of lipid modification is necessary to predict the stability of NLCs. Furthermore, the crystalline state and lipid modification can influence the entrapment efficiency and release kinetics [138]. Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) are commonly used techniques for structural characterization of particles and provide valuable information with respect to crystallinity and polymorphism.

4.5.5 Other colloidal forms and dynamic phenomena

The presence of various colloidal forms such as supercooled melts, micelles, mixed micelles, liposomes etc. is an important factor which can affect stability, drug incorporation capacity and drug release of NLCs. Among other techniques, magnetic resonance techniques such as nuclear magnetic resonance (NMR) and electron spin resonance (ESR) are important tools to evaluate the presence of other colloidal structures. NMR signals can be attributed to the presence of particular molecules or their segments and provide a valuable information on the arrangement of molecules and

their environment. On the other hand, ESR study the interactions between the active principle and the lipids [129].

4.5.6 Encapsulation efficiency

Encapsulation efficiency (EE) is an important factor to optimize in the synthesis process, due to its impact in the release of the active principle, as well as the profitability of the synthesis. EE refers to the percentage of drug that is trapped in the nanoparticle and reflects the effectiveness of the NLC formulation. Lipids with imperfections in their crystal structure usually show higher EE, as it is the case for NLCs thanks to the liquid lipids that increase the number of imperfections [139]. As an example, Tran et al (2014) investigated the effect of NLCs prepared by hot homogenization, using Compritol 888 ATO as a solid lipid, Labrafil M 1944CS as a liquid lipid, and soya lecithin and Tween 80 as emulsifiers over the oral bioavailability of fenofibrate. The drug entrapment efficiency was 99% with a loading capacity of 9.93±0.01% (w/w) [140]

4.5.7 In vitro release

The drug release behavior of NLCs is dependent on the proportion of oil, production temperature and emulsifier concentration. Drug release from NLCs can either be controlled by diffusion of the drug or erosion of the matrix depending upon whether the drug is entrapped in the core of the NLCs, in the shell, or in the matrix. In most of the cases, release of the drug is controlled by the slow dissolution rate in the aqueous environment and the degradation rate of the lipids [129].

4.6 Applications in drug delivery

4.6.1 Topical delivery

NLCs have various advantages over conventional creams and emulsions in terms of providing controlled release, protection of the active component, enhanced permeability into the skin and minimal skin irritation, and have been reported to have better skin targeting efficiency, faster onset, prolonged release, negligible skin irritation and a good safety profile for various therapeutic conditions [129]. Some examples for

NLCs in topical drugs are dodophyllotoxin for warts [141], and anti-inflammatory drugs such as flurbiprofen [142], indomethacin and celecoxib [143].

4.6.2 Oral delivery

Oral route is the most preferred route for the administration of drugs. However, some drugs have solubility issues and are poorly bioavailable due to the presence of various physical, chemical and enzymatic barriers in the gastrointestinal tract [129]. NLCs have been used for oral administration with the aim to enhance oral bioavailability by enhancing the uptake of drug via the intestinal membrane and bypassing the first pass metabolism [144]. NLCs containing oridonin glycerin monostearate, octadecylamine and soybean lecithin S100, and synthesized by the HPH method, generated a relative bioavailability of 171.01% [145]. A similar research was conducted to develop an NLC formulation for vinpocetine. NLCs were prepared by the high-pressure homogenization method. The average encapsulation efficiency was 94.9 ± 0.4% and the relative bioavailability was 322% [146].

4.6.3 Ocular delivery

NLC formulations have been investigated for their potential as an ocular delivery system as they can enhance corneal permeation and thereby improve bioavailability in addition to being safe, non-invasive and patient compliant [129]. NLC formulations have been investigated for the treatment of various disorders of the eye such as inflammation, infections, glaucoma and also disorders affecting the posterior segment of the eye [147].

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Chapter I

Volatile amphiphile-based supramolecular solvents for reducing phospholipid-based matrix effects in LC-MS/MS

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THE USE OF A RESTRICTED ACCESS VOLATILE SUPRAMOLECULAR SOLVENT FOR THE LC/MS-MS ASSAY OF BISPHENOL A IN URINE WITH A SIGNIFICANT REDUCTION OF PHOSDPHOLIPIDS-BASED MATRIX EFFECTS

José Ángel Salatti-Dorado, Noelia Caballero-Casero, María Dolores Sicilia, María Loreto Lunar and Soledad Rubio*

HIGHTLIGHTS

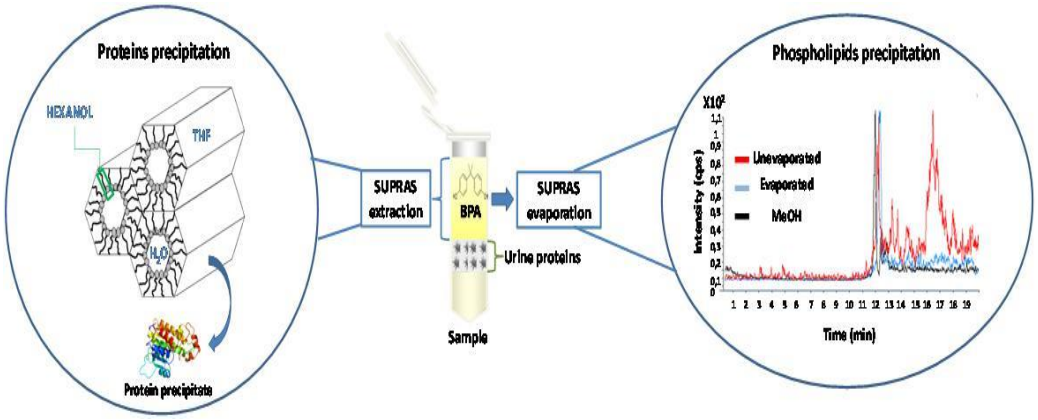
A sample treatment platform is designed for matrix-effect removal in LC-MS bionalysis

It is based on the use of restricted access-volatile supramolecular solvents

Proteins and phospholipids are removed from biological samples by precipitation

Removal of ionization suppression is proved for quantification of BPA in urine by LC-(ESI-)-MS/MS

GRAPHICAL ABSTRACT



KEYWORDS

Supramolecular solvent-based microextraction
Liquid chromatography/ tandem mass spectrometry
Urine samples
Protein/ phospholipid removal
Bisphenol A

ABSTRACT

Restricted access-volatile supramolecular solvents (RAM-VOL-SUPRAS) are here proposed as a new strategy for the quick removal of protein and phospholipids and efficient analyte extraction in LC-MS bioanalysis. Quantification of bisphenolA in urine was selected to prove the suitability of this approach for the intended purpose. RAM-VOL-SUPRAS were spontaneously synthesized in urine by addition of hexanol (83 μL) dissolved in THF (150 μL). SUPRAS composition was environment-dependent and an equation for prediction of SUPRAS volume under given experimental conditions was proposed. Urinary proteins were removed by flocculation by the combined action of THF and hexanol. Phospholipids were extracted in the SUPRAS by the formation of mixed aggregates with hexanol and precipitated as the SUPRAS extract (75 μL) was evaporated to dryness. BPA, re-extracted from the residue, was analysed by LC-(ESI)-MS/MS. Removal of phospholipids by precipitation was proved by monitoring them in both evaporated and unevaporated urine SUPRAS extracts by LC-MS. This removal led to significant reduction in matrix-effects in the determination of BPA. The method quantification limit in urine was 0.025 ng mL^{-1} and the repeatability for 0.4 ng mL^{-1} of BPA, expressed as relative standard deviation, was 4.5%. Concentrations of BPA in the urine samples analysed were in the range 0.357-1.58 ng mL^{-1}. Recoveries were within the range 96-107%. This new approach for sample treatment in bioanalysis, based on the simplicity of dual precipitation of proteins and phospholipids, allows obtaining much cleaner extracts than conventional procedures.

INTRODUCTION

One of the major concerns in LC-MS bioanalysis, especially when using electrospray ionization (ESI), is addressing matrix effects produced by endogenous phospholipids, not only because of their great influence in analyte response but also because of the difficulty in their removal from samples [1].

There are two common structural classes of phospholipids in biological matrices, namely glycerophospholipids [e.g phosphatidylcholine (PC), lysophosphatidylcholine (lyso PC), phosphatidylethanolamine (PE), etc] and sphingomyelins (SM). Their concentration in these matrices can vary greatly between subjects and time of collection. PC is the class of phospholipid that occurs in the largest proportion in human plasma and urine [2,3].

Phospholipids cause ionization suppression in the LC-ESI-MS analysis of co-eluting compounds owing to their enrichment at the surface of the droplets during the liquid/gas-phase ion transfer, which inhibits the ejection of analyte ions trapped inside the droplets [4]. Phospholipids can also influence the assay performance of non-coeluting compounds present in the sample owing to their strong retention on the chromatographic column, which can produce retention time shifts, increased baselines and divergent calibration curves [5,6].

Because of their amphiphilic nature, none of the typical sample treatments applied to biological samples (i.e. protein precipitation, PPT; liquid-liquid extraction, LLE, and solid phase extraction, SPE) offers enough selectivity to remove phospholipid-based matrix effects [1]. PPT removes a fraction of the phospholipid content, the extent of removal depending on the organic solvent used (e.g. methanol extracts contain more phospholipid compared to acetonitrile, tetrahydrofuran or ethanol extracts) [7-9]. LLE is well suited for the extraction of lipophilic compounds but phospholipids will co-extract with analytes due to the hydrophobicity of the two fatty acyl groups [10]. Recovery of polar analytes in LLE can increase by extraction with the mixtures of halogenated solvents and alcohols but these mixtures also have high affinity for phospholipids [11]. Compared to PPT and LLE, SPE is considered much more effective for recovery of analytes and reduction of phospholipid-based matrix effects [12,13]. Both reversed phase and mixed mode strong anion exchange are commonly used as sorbents in SPE; in the former, phospholipids will co-extract with analytes due

to their hydrophobic tail while in the latter, phospholipids will co-extract due to their zwitterionic polar head group [4].

In the last few years, the combination of PPT and SPE has been increasingly used as a strategy for reducing phospholipid-based matrix-effects [14-18]. The approach has been commercialized by several companies (e.g. Hybrid SPE™ from Sigma Aldrich, Ostro™ from Waters, Captiva™ ND from Agilent and Phree™ from Phenomenex) and it is available in the 96-well plate format. PPT is based on organic solvents while SPE is based on different sorbents (e.g. zirconia-coated silica in Hybrid SPE™, C18 in Ostro™, etc.). The PPT-SPE extracts contain lower phospholipids concentrations as compared to the use of single PPT or SPE, but the problem of how to avoid the retention of analytes in the sorbent causing low recoveries remains. Overall, reliable bioanalyses can be achieved with proper sample preparation and the use of isotopically labeled standards. Sometimes, however, the high variability of the matrix composition makes the use of standard addition calibration necessary [19,20].

In this manuscript, a new sample preparation platform designed for the removal of matrix-effects in LC-MS bioanalysis is proposed. It is based on the dual precipitation of proteins and phospholipids by using restricted access, volatile supramolecular solvents (RAM-VOL-SUPRAS).

Supramolecular solvents are nanostructured liquids produced from colloidal solutions of amphiphiles by spontaneous processes of self-assembly and coacervation [21,22]. The high number of binding sites that they offer to solutes (e.g. concentration of amphiphiles in the SUPRAS is usually in the interval 0.1-1 mg μL^{-1}) and the regions of different polarity present in their nanostructures, derived of the amphiphilic character of the molecules making them up, render SUPRASs excellent extractants for solutes in a wide polarity range [23,24].

Amphiphiles in the SUPRAS nanostructures are held together by non-covalent interactions, which makes them environment responsive and opens the door to the design of tailored solvents [25]. Thus, SUPRAS with restricted access properties (RAM-SUPRAS), that can extract low molecular weight solutes while excluding macromolecules through chemical (e.g. proteins precipitation) and physical (size exclusion of carbohydrates, humic acids, etc) mechanisms, have been reported [26]. They are made up of inverted hexagonal aggregates of alkanols (C8-C14), where the alcohol groups surround aqueous cavities and the hydrocarbon chains are dissolved in

THF. The size of the aqueous cavities, and thus their exclusion ability, can be tailored by proper selection of the environment (i.e. THF/water ratio) for coacervation. Proteins are precipitated by the action of the THF and alkanol. RAM-SUPRAS have been applied to the efficient extraction of a variety of solutes and sample cleanup in food [27-29] and environmental [30,31] analysis.

A handicap for the application of RAM-SUPRAS to LC-MS bioanalysis is that, although a fraction of phospholipids is removed during PPT, the remaining fraction forms mixed aggregates with the amphiphiles in the SUPRAS and co-extracts with analytes. So, in order to develop generic sample treatments for bioanalysis based on SUPRASs, the complete removal of phospholipids is required. With this aim, in this work, a new RAM-SUPRAS based on hexanol is synthesized, characterized and applied to the simultaneous extraction of bisphenol A in urine and the removal of endogenous proteins and phospholipids. To the best of our knowledge no solvents with the ability to remove both types of interferences in biological analysis have to date been reported. Below, the most salient results of this study are described and discussed.

EXPERIMENTAL

Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Tetrahydrofuran (THF), ammonia and acetic acid were purchased from Panreac (Barcelona, Spain) and 1-hexanol from Merk (Darmstadt, Germany). LC grade methanol was supplied by VWR Chemicals (LlinarsdelVallès, Barcelona, Spain). Bisphenol A (BPA) was obtained from Fluka (Madrid, Spain) and isotopically labeled bisphenol A ($^{13}C_{12}$- BPA, 100 mg L^{-1} in acetonitrile) from IS Cambridge Isotope Laboratories (Andover, USA). β-glucuronidase from Helix pomatia, H1 (1634000 units g^{-1} β-glucuronidase; 14000 units g^{-1}sulfatase), was purchased in Sigma Aldrich (Steinheim, Germany). Stock solutions of BPA (0.5 g L^{-1}) and $^{13}C_{12}$- BPA (1.2 mg L^{-1}) were prepared in methanol and stored at 4°C. Working solutions of BPA were made by appropriate dilution of the stock solution with methanol. A β-glucuronidase/sulfatase solution (30000/257 units mL^{-1}) was prepared by dissolving the solid in 1M NH_4Ac buffer (pH 5) and stored under dark conditions at 4ºC.

Apparatus

The separation and quantitation of BPA was accomplished by using a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex 4000QTRAP, Foster City, CA, USA) coupled to a liquid chromatograph (Agilent HP 1200 series, Palo Alto, CA, USA) with a TurboIonSpray (TIS) interface. All data were acquired and processed using the Analyst 1.5.1 Software. The stationary phase was an ACE C18-PFP column (3 μm, 150 mm×3.0 mm) from Advanced Chromatography Technologies (Aberdeen, UK). It was preceded by aguard cartridge (ACE C18-PFP; 3μm, 10 mm × 3 mm) in order to protect the analytical column. A coloumetric Karl Fischer titrator from Methrom (Herisaus, Suize) and a gas chromatograh with flame ionization detector (GC-FID) from Thermo Quest (Milan, Italy) were used for the determination of water and hexanol contents in the SUPRAS, respectively. The volume of SUPRASs obtained under different experimental conditions was measured using a digital calliper from Medid Precision, S.A. (Barcelona, Spain). Two mL-microtubes Safe-Lock from EppendorfIbérica (Madrid, Spain), a vortex shaker from ReaxHeidolph (Schwabach, Germany) with an attachment for 10 tubes, and a high speed brushless centrifuge MPW-350R from MPW Medical Instruments (Warschow, Poland) were used for sample preparation. A digitally regulated centrifuge, Mixtasel from JP-Selecta (Abrera, Spain) was used for urine centrifugation before sample treatment. An incubator VorTemp 1550 from Labnet International (Edison, NJ, USA) adjusted at 37ºC was used to carry out the enzymatic hydrolysis of BPA conjugates. A sample evaporator/concentrator (SBHCONC/1 and SBH130D/3, Stuart, France) was used for the evaporation of the SUPRAS extract.

Hexanol-based SUPRAS characterization

SUPRASs were synthesized through spontaneous self-assembly and coacervation of hexanol/THF/water and hexanol/THF/urine ternary mixtures. Phase diagrams were constructed by mixing the three components at different percentage (v/v) in order to delimit the region for SUPRAS formation. The SUPRASs produced in this region were always made up of hexanol, THF and water. The relative proportion of these components in the SUPRAS (%, v/v) as a function of the content of hexanol (1-10%, v/v) and THF (1-50%, v/v) in the synthetic solution was determined. Water and hexanol were analyzed by coloumetric Karl Fisher titration and GC-FID, respectively,

while the THF content was calculated by difference. The volume of SUPRAS formed from synthetic solutions containing 1-10% (v/v) of hexanol and 1-50% (v/v) THF was also measured (n=50). Non-linear regression was used to fit a model for the prediction of the volume of SUPRAS as a function of the composition of the ternary mixture. The statistic program Statgraphics Centurion XV was used for this purpose.

Determination of total BPA in urine

Samples

The spot urine samples analysed belonged to the Sabadell birth cohort, which includes samples from pregnant women at around 12 and 32 weeks of gestation and their children obtained at 4 years of age [32]. These samples were kept in polypropylene tubes at -20°C until use. On the other hand, a urine pool was created by the combination of nine individual specimens from volunteers (men and women) for characterization of the SUPRASs and optimization of the extraction of BPA and interference removal. Each individual contributed the same volume to the pool and aliquots of about 45 mL were stored in glass bottles and frozen at -20 ºC until use.

Control of background contamination

Because of the ubiquity of BPA, strict measures were established to prevent BPA background contamination coming from LC tubing, solvents and labware. Glassware was successively cleaned with water and detergent, distilled water and methanol (twice each step). Eppendorf microtubes were rinsed with methanol several times before their use. The interference of the potential BPA leached from the plastic components of the LC system was removed by placing a symmetry C18 column (3.5 μm, 75 mm × 4.6 mm) from Waters (Milford, MA, USA) in the line of the mobile phase before the injection valve. In this way, the BPA leached, if any, eluted later in the chromatogram. BPA free water was obtained by filtration of ultrapure water (Merck, Darmstadt, Germany) through Empore SDB-XC disks (AnálisisVínicos, Tomelloso, Spain). Quality controls were routinely run with each batch of samples and were always below the quantification limit.

Hydrolysis of BPA conjugates

Frozen urine samples were allowed to thaw at room temperature and centrifuged (20 min at 2.000 x g) to remove suspended material. Then, sample aliquots (1.4 mL) were transferred into 10 mL glass tubes and spiked with 1.7 ng mL^{-1} of $^{13}C_{12}$- BPA. After gentle mixing, the hydrolysis of BPA conjugates was performed by adding 23 µL of the β-glucuronidase/sulfatase solution (30.000/257 units mL^{-1} in 1 M ammonium acetate buffer pH 5.0). It was allowed to proceed overnight at 37 °C. Next, the samples were vortex-shaken for 1 min and centrifuged (2.000 x g, 10 min).

RAM-VOL-SUPRAS-based microextraction and protein and phospholipid removal

Extraction of BPA from urine was performed by adding 1.267µL of the hydrolyzed samples into 2 mL polypropylene centrifuge microtubes containing hexanol (83 µL) dissolved in THF (150 µL). The SUPRAS formed spontaneously by coacervation of hexanol in the presence of the urinary water. The mixtures were vortex-shaken for 7 min and then centrifuged for separation of the SUPRAS from protein precipitate (14.160 x g, 30 min, 15 ºC). After that, 75 µL of the SUPRAS extract were withdrawn with a glass microsyringe, transferred into glass tubes and evaporated to dryness at 60 °C, under a gentle nitrogen stream. The residue, containing the phospholipid precipitate was treated with 300 µL of methanol:water 50:50 (v/v) and shaken for 5 seconds by hand to dissolve BPA. Then, the extract was transferred to an autosampler glass vial with insert. Samples were capped with butilo/PTFE septum barriers (Análisis Vínicos, Tomelloso, Spain) and subjected to LC/MS/MS analysis. A scheme of the whole procedure is shown in Figure 1.

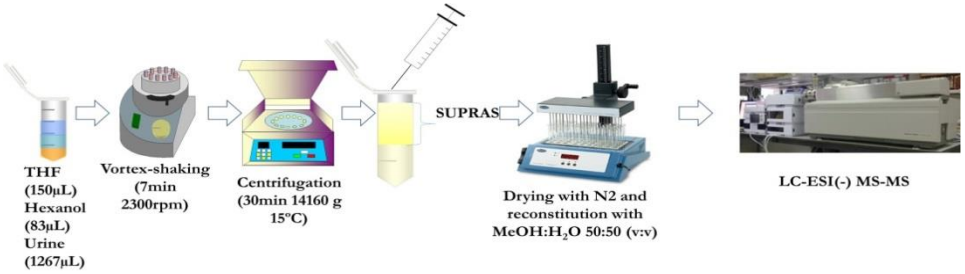


Figure 1. Scheme for the LC-(ESI)MS/MS quantification of BPA in urine, previous BPA extraction and protein/phospholipid removal using RAM-VOL-SUPRAS

Quantification of BPA by LC (ESI-) MS-MS

Quantification of total BPA was carried out by liquid chromatography coupled with tandem mass spectrometry through a turbo spray interface operating in the negative ion mode. The mobile phase consisted of water and methanol at a flow rate of 0.4 mL min^{-1}. The elution program was as follows: linear gradient from 40% to 60% of methanol for 20 min and then reverting to initial conditions allowing 10 min for column stabilization. The eluates from the analytical column were diverted by the switching valve to waste, except for the elution window from 9 to 11 min. The injection volume was 10 μL and the temperature for the analytical column was set at 35 ºC.

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. For BPA, MRM transitions were (m/z) 227→212 (quantifier ion) and 227→133 (qualifier ion). $^{13}C_{12}$-BPA, used as internal standard, was monitored at the transitions (m/z) 239→224 (quantifier ion) and 239→139 (qualifier ion). The dwell time was set up at 100 ms. The TIS source values were adjusted as follows: probe vertical y-axis position, 2 mm; probe horizontal y-axis position, 6 mm; curtain gas (N_2), 27 psi; ion source gas 1 (nebulizer gas), 70 psi; ion source gas 2 (turbo gas), 50 psi; temperature of the turbo gas, 600 ºC; ion spray voltage, -4.500 V; entrance potential, -10 V; and declustering potential, -95 V. Parameter values for the analyzer were as follows: 1.0 unit resolution for the first and third quadrupoles; collision gas, 3.0 $x10^{-5}$ Torr; collision energy, -26 V; and collision cell exit potential, -5 V.

Solvent calibration was run from methanol:water (50:50) solutions containing BPA in the interval 0.025-500 ng mL^{-1} and 5.0 ng mL^{-1} of $^{13}C_{12}$-BPA. Correlation between BPA/internal standard peak area ratios and concentrations of BPA was determined by linear regression and 1/x weighted calibration.

Monitoring of phospholipids in urine

The efficiency of the procedure described in section 2.4.4 to remove phospholipids from urine was checked by monitoring them in evaporated and unevaporated SUPRAS extracts obtained from a urine pool sample. Monitorization was carried out by LC coupled to a triple quadrupole mass spectrometer by an electrospray interface operating in the positive ion mode, according to a method previously reported [4]. Analyses were made in the scan mode within the range 430-850 *m/z*. The mobile

phase consisted of 1 mM ammonium acetate with 0.5% formic acid (eluent A) and MeOH (eluent B). The elution program was amended slightly as follows: 20 % of eluent B for 4.5 min, linear gradient for 2.5 min up to 70 % and then 95 % for 8 min. Finally, it was reverted to initial conditions allowing 5 min for column stabilization. The injection volume was 10 μL and the stationary phase was an ACE C18-PFP column (3 μm, 150 mm×3.0 mm) from Advanced Chromatography Technologies (Aberdeen, UK).

RESULTS AND DISCUSSION

Synthesis and characterization of restricted access-volatile supramolecular solvents (RAM-VOL-SUPRAS)

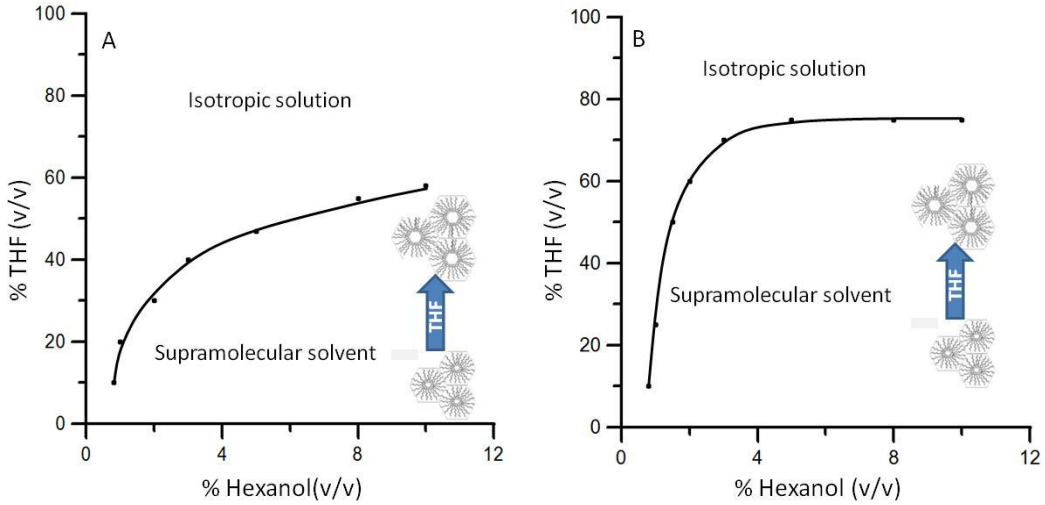
In order to develop generic sample treatments in LC-MS bioanalysis, hexanol was selected as amphiphile for the design of SUPRASs able to remove endogenous proteins and phospholipids.

It was checked that hexanol, similarly to alkanols of longer hydrocarbon chains (C8-C14) [26] formed SUPRAS in binary mixtures of THF and water. Thus, hexanol formed reversed aggregates in THF and coacervated under addition of water, despite its high solubility in this solvent (5.9 g L^{-1}) compared to that of octanol (0.46 g L^{-1}). This meant that amphiphile-amphiphile interactions were still strong enough to predominate over amphiphile-solvent interactions. Coacervation of hexanol also occurred in urine because of the high water content of this biological matrix (i.e. ~95%), so SUPRAS could be spontaneously synthesized in the samples.

Figure 2 shows the phase diagrams obtained from ternary mixtures of (A) hexanol-THF-water and (B) hexanol-THF-urine, at room temperature. The study of percentages of hexanol was restricted to those of greater analytical interest (e.g. below 10%) because higher concentration factors can be achieved at the lowest concentrations of amphiphile. The percentages of water and urine are not represented in these diagrams but they can be easily calculated from the percentages of hexanol and THF.

Two regions were visible to the naked eye in both diagrams: the SUPRAS and isotropic solution regions. Their boundary was hexanol-dependent for percentages below around 3%. Above this value, the behavior was different in water (hexanol-dependent) and urine (hexanol-independent). Because of the higher density of human

urine (e.g. 1.015-1.020 g cm^{-3} at 20ºC) compared to distilled water (e.g. 0.998 g cm^{-3}at 20 ºC), SUPRAS immiscibility was favored in urine and consequently the region for SUPRAS formation became wider. This behavior was without analytical significance since the highest concentration factors are achieved at the lowest percentages of THF. In both cases, SUPRAS densities were lower than those of the solutions in which they formed.



Fugure 2. Phase diagrams for ternary mixtures of (A) hexanol-THF-water and (B) hexanol-THF-urine. The hexagonal insets in the figure show the environment-responsive character of the nanostructures making up the SUPRASs

The volume of SUPRAS obtained was linearly and exponentially dependent on the percentages of hexanol and THF, respectively, in the synthetic (urine or water) solution. This means that SUPRAS composition depended on the environment (e.g. THF:water or THF:urine ratios) in which they formed. A general equation was derived to predict the volume of solvent produced from given experimental conditions. For this purpose, two sets of SUPRASs were prepared in water and urine using different hexanol (1-10%, v/v) and THF (1-50%, v/v) concentrations within the region of coacervation. Nonlinear regression was used to fit a model to the data obtained (n_{water} = n_{urine} = 50). The proposed model for predicting the volume of SUPRASs obtained from water or urine (V_{SUPRAS}, µL mL^{-1} solution) was:

$$V_{SUPRAS} = (10.7 \pm 0.3)He^{(0.0330 \pm 0.0007)THF} \quad (1)$$

where hexanol (H) and THF percentages (v/v) in the bulk solution are the independent variables. The regression coefficient was 98.93%, thus indicating a good capability of prediction of this equation. So, the same volume of SUPRAS was obtained from water and urine, that indicating that SUPRAS composition and structure were not altered by

urine matrix components. From this equation, the maximum concentration factors that can be achieved with hexanol-based SUPRASs under given conditions can be known a priori and this makes easier method selection and optimization. Taking into account this equation, the highest concentration factors will be obtained using low percentages of hexanol and THF.

Table 1 shows the chemical composition of SUPRASs obtained under different experimental conditions. SUPRAS composition was amphiphile-independent and environment-dependent. The percentages of hexanol in the SUPRAS were practically constant for the whole range of percentages of amphiphile tested (1-10%, THF=10%, v/v). In all the cases, the incorporation of the amphiphile to the SUPRAS was above 90%. Contrarily, SUPRAS composition was highly dependent on the THF:water ratio in the synthetic solution. Thus, the increase in the percentage of THF (1-40%, v/v) gave SUPRAS increasingly diluted in hexanol and increased content in THF and water. The hexanol incorporated to the SUPRAS also progressively decreased. The results in this table, similarly to those obtained from volume studies confirm that, from an analytical point of view, the best SUPRASs for solute extractions will be those obtained from low percentages of THF since they provide the highest number of binding sites and concentration factors.

An important conclusion that can be inferred from the increased content of water in the SUPRAS as the THF:water ratio increases in the synthetic solution is that the size of the aqueous cavities in the SUPRAS increases (see Figure 2) and, consequently, these solvents can be tailored to behave as restricted access SUPRAS (RAM-SUPRAS), similarly to the behavior of alkanols of longer hydrocarbon chains [26].

The hexanol-based RAM-VOL-SUPRASs offer different types of interactions for solute solubilization; namely hydrogen bonding and polar interactions in the polar groups and dispersion interactions in the hydrocarbon chains. These interactions can work in a cooperative manner thus increasing the potential of SUPRASs to be highly efficient extractants for solutes in a wide polarity range.

Table 1. SUPRAS composition and percentage of hexanol incorporated into the SUPRASs obtained at different percentages of THF and hexanol in the synthetic solutions

Synthetic solution	SUPRAS composition (%, v/v)			% HEXANOL incorporated in the SUPRASs
	THF	H_2O	Hexanol	
Hexanol[a] (%, v/v)				
1	35.7	3.3	60.7	91.0
2	32.7	3.3	64.4	95.3
5	31.5	3.5	65.3	96.7
6	32.2	3.8	64.4	95.3
7	31.7	3.3	64.6	96.7
10	31.5	3.6	65.4	96.7
THF[b] (%, v/v)				
1	5.8	2.2	91.8	102.0
5	17.8	3.3	78.5	99.7
10	32.2	3.8	64.4	95.3
20	52.4	4.6	43.0	89.0
30	66.5	6.5	27.1	77.8
40	73.8	9.2	16.9	68.1

[a] THF= 10 % (v/v) ; [b]Hexanol = 6 % (v/v)

RAM-VOL-SUPRAS-based protein and phospholipid removal

Protein removal during the formation of RAM-SUPRAS from alkanol-THF-water mixtures has been widely proved in food samples and protein standard solutions [27-29]. Proteins precipitate in the presence of THF and alkanols and give a white solid layer between the SUPRAS extract and the sample solution after centrifugation. It was checked that proteins in the urine behaved similarly under addition of hexanol and THF and consequently, their interference was removed during BPA extraction.

Regarding phospholipids, preliminary experiments indicated that there was severe ionization suppression or enhancement in the determination of BPA in some urine samples when SUPRAS extracts were directly analyzed by LC-(ESI)MS/MS. Matrix-effects were highly specimen-dependent and not corrected by the use of isotopically labeled BPA as internal standard. Thus, the analysis of three different BPA-fortified (1 ng mL^{-1}) and unfortified urine SUPRAS extracts gave values for signal suppression or enhancement (SSE) of 0, 0 and 129%, respectively (SSE is referred to as an absolute

matrix effect; percentages above and below 100 indicate ion enhancement and suppression, respectively [33]).

Removal of phospholipids was tried by their precipitation after volatilization of the SUPRAS extract under a nitrogen stream. The impact of this removal on matrix-effects was investigated by comparing the SSE values for evaporated and unevaporated BPA-fortified (1 ng mL^{-1}) and unfortified SUPRAS extracts from a urine sample (section 2.4.1). The residue of the evaporated fraction was treated with 300 μL of methanol:water 50:50 (v/v) for BPA re-extraction. The values of SSE obtained from the measurement of evaporated and unevaporated extracts were 103 and 0, indicating that evaporation of the SUPRAS extract was an effective strategy for removal of matrix effects.

In order to determine if matrix effects were caused by phospholipids, monitoring of these endogenous compounds by LC-MS (section 2.5) was carried out in both evaporated and unevaporated SUPRAS extracts from a urine pool sample (section 2.4.1). Figure 3(A-F) shows some of the most representative results obtained in this study. For comparison, the monitoring of methanol is also included in all the LC-MS chromatograms.

Total ion chromatograms (e.g. Figure 3A) were run from 430 to 850 m/z, interval where the m/z of the precursor ions $[M+H]^+$ corresponding to the most important phospholipids present in biological matrices are included [4]. Reduction of the signals obtained in the intervals 2.5-8 min and 13-20 min was clearly observed for evaporated SUPRAS extracts compared to the unevaporated ones.

Extracted ion chromatograms for specific phospholipids, run at the m/z values corresponding to their precursor ions $[M+H]^+$, are shown in Figure 3(B-F) (e.g. phospholipids from the phosphatidylcholine (B), lysophosphadidylcholine (C), sphingomyelin (D-E) and phosphatidylethanolamine (F) classes [4]). A high reduction or complete removal of the different phospholipids present in the extracts was always obtained as the SUPRAS was evaporated. These results indicated that phospholipids were precipitated and not re-dissolved as the residue was treated with methanol:water (50:50) for solubilization of BPA and, consequently, phospholipid precipitation is an effective strategy for their removal.

The step for phospholipid removal was optimized in terms of the volume of SUPRAS evaporated, volume of methanol:water (50:50) used for BPA re-dissolution,

and time for BPA re-extraction. In all these experiments, urine SUPRAS extracts, fortified with BPA at the 0.4 ng mL^{-1} level, were used.

Recoveries for BPA were near 100% and independent of the volume of SUPRAS evaporated in the interval investigated (50-75 μL). Evaporation of 75 μL of SUPRAS is recommended because collection of greater SUPRAS volumes from the sample extract (calculated total volume from eq. 1 = 123μL) should be performed carefully in order not to collect urine sample. The volume of methanol:water (50:50) used for BPA re-dissolution was investigated in the interval 150-300 μL. Recoveries for BPA were 37.6±0.1, 44.7±0.4, 91.7±0.3 and 103±1% as the re-extraction was carried out with 150, 200, 250 and 300 μL of the solvent mixture, respectively, so the latter volume is recommended. Extraction of BPA was quantitative after about 5 s of gently manual stirring of the residue with 300 μL of methanol:water (50:50). Vigorous stirring or longer extraction times should be avoided since, as it has been previously reported [34], extraction time plays an important role for the specific extraction of target analytes compared to matrix components (i.e. matrix compounds diffuse slower into the extraction solvent).

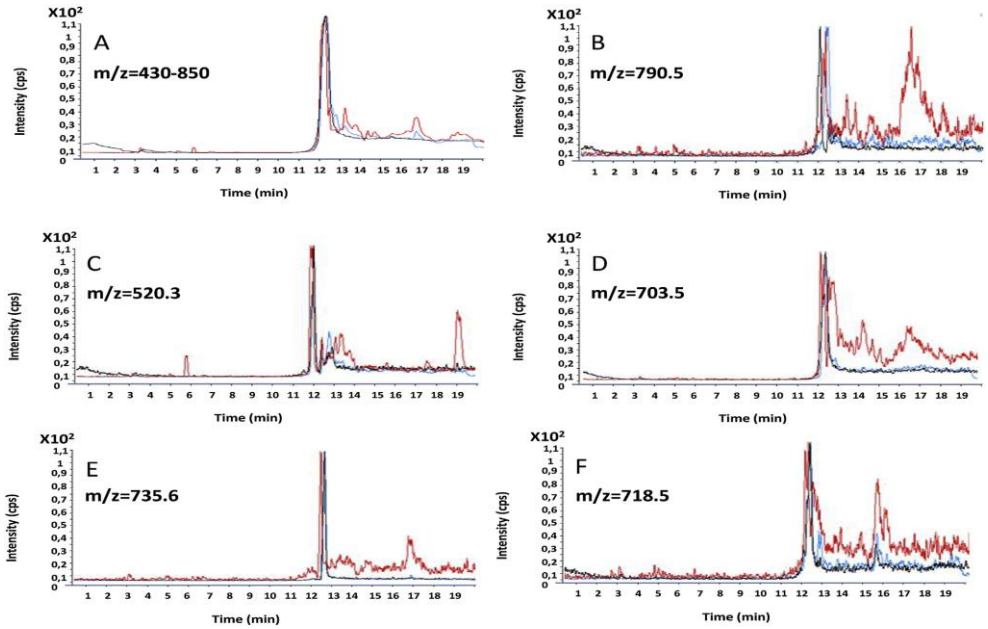


Figure 3. (A) Total ion chromatogram and (BeF) extracted ion chromatograms obtained by monitoring phospholipids in (red) unevaporated and (blue) evaporated urine SUPRAS extracts by LC-MS. Black chromatograms correspond to the monitoring of pure methanol. The m/z values shown correspond to phospholipids belonging to the (B) phosphatidylcholine, (C) lysophosphadidylcholine, (DeE) sphingomyelin and (F) phosphatidylethanolamine classes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

RAM-VOL-SUPRAS-based BPA extraction

According to the interactions offered by the SUPRAS and the structure of BPA, the most probable binding sites for solubilization of the analyte in the solvent are both the polar groups (i.e. by hydrogen bonding and polar interactions) and the hydrocarbon chains (i.e. by dispersion interactions), working in a cooperative manner.

Extraction of BPA was optimized in terms of SUPRAS volume (i.e. varying the percentage of hexanol while keeping constant the THF/urine ratio), SUPRAS composition (i.e. varying the THF/urine ratio while keeping constant the content of hexanol), and pH and time for extraction. A urine pool (section 2.4.1), both unfortified and fortified with BPA 0.4 ng mL^{-1}, was used for this purpose. The experimental conditions tested were: 4.5-10% (v/v) of hexanol, 5-40% (v/v) of THF, pH 3-10 and extraction time 1-10 min. Extractions were carried out according to the procedure specified in section 2.4.4. Selection of the optimal conditions was based on the recoveries obtained for BPA. Experiments were made in triplicate.

Table 2 shows the recoveries obtained as a function of both hexanol and THF percentages. The volumes of SUPRAS formed in the sample, calculated from equation 1, are also included. Hexanol was proved to be an excellent extractant for BPA and quantitative recoveries were obtained from 5.5% that was selected as optimal. Recoveries for BPA were SUPRAS composition-independent in the interval 10-30% of THF. The minimal percentage of THF giving quantitative recoveries (i.e. 10%) was selected in order to minimize organic solvent consumption in the synthetic solution (i.e. 150 μL per sample).

The pH did not influence BPA recoveries in the interval investigated (i.e. 3-10). The analyte was extracted in the unionized form (i.e. pK_a BPA = 10.3±0.1). The equilibrium conditions for extraction were reached after 7 min of vortex-shaking of the sample (e.g. BPA recoveries were 93±9, 96±8, 102±4 and 103±4 at 1, 5, 7 and 10 min, respectively).

Table 2. Mean percent recoveries obtained for bisphenol A as a function of the percentage of hexanol and THF used for the formation of the SUPRAS

Variable	SUPRAS Volume[a] (µL)	Recovery[b]±s[c](%)
Hexanol[d] (%, v/v)		
4.5	100	88.2±0.9
5.5	123	98.6±0.8
8	178	97±3
10	222	107±4
THF[e] (%, v/v/v)		
5	103	75±1
10	123	98.6±0.8
20	171	101±2
30	238	98±1
40	333	68.5±0.9

[a]Calculated from equation 1, section 3.1; [b]Spiking level: 0.4 ng mL^{-1} of BPA; [c]Standard deviation,n=3; [d]THF=10 % (v/v); [e]Hexanol = 5.5 % (v/v)

Analytical performance

The slope and intercept of the calibration curve, run from methanol:water (50:50) solutions containing BPA and $^{13}C_{12}$-BPA (see section 2.4.5), were 0.1900±0.0008 mL ng^{-1} and 0.07±0.12, respectively. The correlation coefficient was 0.9998 indicating good fit. Method detection (MDL) and quantification (MQL) limits were calculated from the independent analysis of six urine samples, according to the procedure described in section 2.4, and considering a signal to noise ratio of 3 and 10, respectively. Since no blank urine samples could be obtained, an estimate of the background signal was made at a representative part of the readout, adjacent to the analyte signal. The values obtained for MDL and MQL were 0.015 and 0.025 ng mL^{-1}. Similar values of MQLs have been previously reported for BPA in urine (0.01-0.05 ng mL^{-1}), the sensitivity mainly depending on the LC/MS/MS used [35].

The selectivity for the quantification of BPA in urine was assessed by comparing the slope of the calibration curve obtained from standards in 50:50 methanol:water (0.1900±0.0008 mL ng^{-1}) with that run from urine aliquots fortified with known concentrations of BPA in the interval 0.025-500 ng L^{-1} and analysed using the whole procedure (see section 2.4). The slope of calibration curve for the fortified urine sample

was 0.186±0.006 mL ng^{-1}. The difference between both slopes was found to be not statistically significant by applying a Student's t test [36]. The experimental t-value, 2.69, was below the critical t-value (3.05, at the significant level of 0.01).

The precision, evaluated in terms of repeatability, was calculated from the analysis of eleven aliquots of a pooled urine sample fortified with 0.4 ng mL^{-1} of BPA, by following the procedure described in section 2.4. The repeatability, expressed as relative standard deviation (RSD), was 4.5%.

Determination of BPA in human urine

The suitability of the proposed method for determining total urinary BPA was assessed by analysing 12 samples from the Sabadell birth cohort (see section 2.4.1). Samples were analysed in triplicate. Table 3 shows the results obtained and the corresponding recoveries for samples fortified with BPA at the level of 0.4 ng mL^{-1}. Concentrations found for BPA were in the range 0.357-1.58 ng mL^{-1}, which was consistent with the values usually reported in the literature [35]. Recoveries were within the range 96-107%, thus proving the accuracy of the method. Figure 4 shows as an example the SRM ion chromatograms for the transitions corresponding to the quantifier (A) and qualifier (B) ions for BPA from a standard solution (1) and a urine sample (2) and for $^{13}C_{12}$-BPA from the same urine sample (3).

The concentrations found for total BPA by the proposed method were compared to those obtained by applying a LC/MS/MS method, based on multimode SPE sample clean-up, to the same urine samples [37]. For this purpose, the regression line approach was applied to the mean concentration (n=3) obtained for each urine sample from the two methods [38]. The slope and intercept of the regression line (X axis: SUPRAS method; Y axis: multimode SPE method) were 0.910 and -0.088, respectively, with 95% confidence intervals of 0.813-1.007 and -0.130-(+0.113). These ranges included the ideal value of 1 and 0 for the slope and intercept, respectively. The correlation coefficient was 0.991. So, no systematic errors were detected for determination of BPA using the RAM-VOL-SUPRAS-based method.

Table 3. Mean concentrations and recoveries, along with the corresponding standard deviations (n=3) found for total BPA in human urine analyzed by the RAM-VOL-SUPRAS-based method

Urine sample	$[BPA]_{total} \pm s^a$ (ng mL^{-1})	Recovery$^b \pm s^a$ (%)
1	0.51±0.01	98.7 ± 0.9
2	1.05±0.02	107 ± 3
3	0.357±0.003	102 ± 1
4	1.36±0.03	107 ± 4
5	1.52±0.01	99.6 ± 0.8
6	1.53±0.01	99.3 ± 0.8
7	0.848±0.009	100 ± 2
8	1.58±0.02	103 ± 2
9	0.90±0.01	98 ± 1
10	0.74±0.02	98.6 ± 0.9
11	1.26±0.02	96 ± 2
12	1.12±0.01	102 ± 3

[a] Standard deviation, n=3; [b] Speaking level: 0.4 ng mL^{-1} of BPA

CONCLUSIONS

The results obtained in this work show that RAM-VOL-SUPRAS have the ability to remove proteins and phospholipids from urine samples and consequently, to eliminate or dramatically reduce matrix-effects in LC-MS/MS analysis of these samples. This approach, based on the precipitation of both endogenous components, has the potential to be a generic platform for sample treatment of biological matrices.

Two of the most important benefits of this approach are its simplicity from a conceptual and practical point of view and its efficiency for interference removal and analyte extraction. Thus, only minute volumes of commercially available reagents (83 μL of hexanol and 150 μL of THF per urine sample) are required for SUPRAS formation, BPA extraction and protein precipitation. High extraction efficiency for solutes in a wide polarity range is expected as a result of the large number of binding sites and different polarity regions present in the nanostructures making up the SUPRASs. Phospholipids precipitation is quickly carried out by evaporation of minute volumes of SUPRAS extracts (75 μL) and their re-extraction is minimized by their slower diffusion into the extraction solvent compared to that of solutes [34]. On the other hand,

conventional and non-expensive equipment is required for sample treatment and several samples can be simultaneously treated. So, the approach is within everyone´s reach.

The approach is not applicable to volatile compounds. However, most of the LC-MS bioanalyses involve polar and non-volatile compounds.

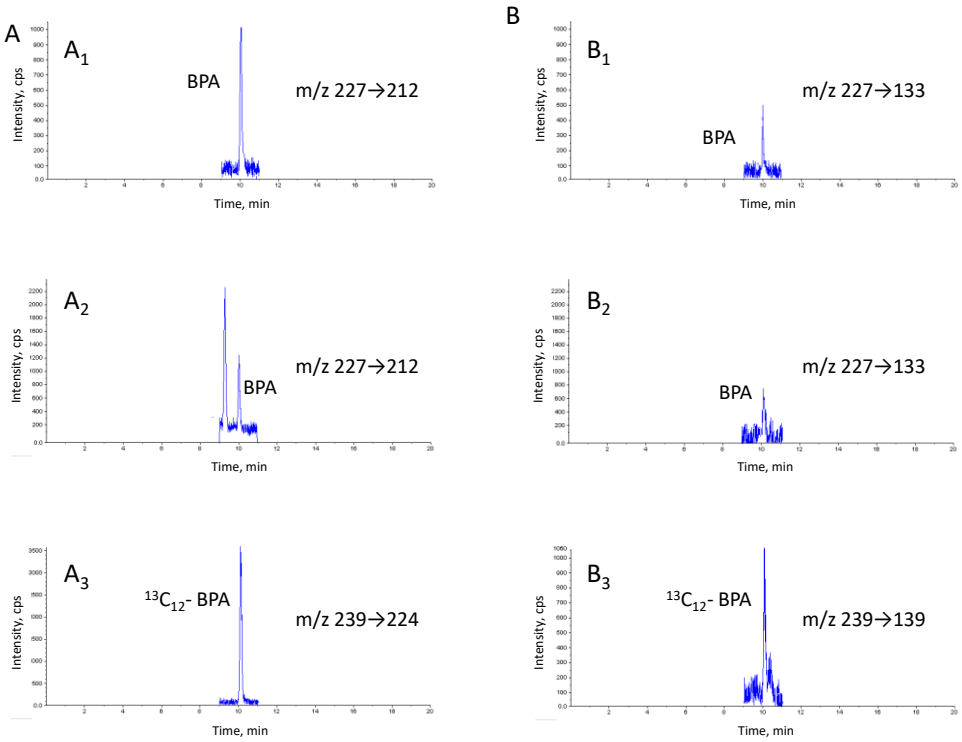


Figure 4. SRM ion chromatograms obtained by LC-(ESI)MS/MS for the transitions corresponding to the (A) quantifier and (B) qualifier ions for BPA from (1) a standard solution and (2) a urine sample and (3) for 13C12- BPA (5 ng mL-1) from the same urine sample

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Chapter II

Supramolecular solvents formed by oligomeric surfactants to reduce the surfactant losses in the equilibrium solution

WATER-INDUCED ENVIRONMENT-RESPONSIVE COACERVATION OF POLY-UNDECYLENIC ACID

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Submitted to Soft Matter

ABSTRACT

We report here the first synthesis of coacervates from ternary mixtures of the oligomeric surfactant poly-undecylenic acid, water and tetrahydrofuran or ethanol. These coacervates were fully characterized regarding phase behaviour, composition, volume and structure. They show a unique property not previously described for any surfactant-based coacervate, mainly: a quantitative incorporation of the oligomer to the coacervate for any synthetic initial conditions. This property extends the versatility of coacervates and may contribute to keep broadening their applicability in additional fields of science. The global composition of the solvent and the size of the coacervate droplets that form it, can be tailored by controlling the environment (specifically, the THF:water and ethanol:water ratio in the bulk solution) for self-assembly. Interestingly, the coacervates formed are highly adaptive and the previous features can all be reversed by modifying the environment. The spontaneous self-assembly of these solvents followed predictable routes, and their composition and volume can be accurately predicted from equations derived in this work.

INTRODUCTION

The interest for coacervation, the phenomenon in which a colloidal or macromolecular solution spontaneously separates in two immiscible liquid phases, one rich (coacervate) and another poor (equilibrium solution) in the colloidal or macromolecular component, has grown steeply in the last decades.[1,2] Coacervation is classified into simple and complex depending on wether a single or two oppositely charged colloidal or macromolecular components are involved. Many areas have benefited from coacervation so far including extraction of organics[3] and inorganics,[4]

cosmetic formulations,[5] protein purification,[6] microencapsulation,[7] wastewater treatment,[8] and so on.

Application of coacervation in extraction processes (e.g. extraction of contaminants in environmental, food or biological samples prior to their chemical analysis,[9] bioactive compounds from vegetal biomass and agro-industrial residues,[2] pollutants removal in water purification,[10] etc.) has largely been based on surfactants.[11,12] Coacervation of surfactants occurs through two sequential self-assembly steps; firstly, they spontaneously self-assemble above a critical aggregation concentration (cac) to give a colloidal solution containing micelles, vesicles, and so on. Aggregation of surfactants is a start-stop process; for typical surfactants, solvophobicity drives aggregation while the stop process emanates from head group-head group repulsion. In order to induce coacervation in these colloidal solutions, surfactant aggregates have to become bigger. Aggregate growth in this step involves reducing the head group-head group repulsions that stopped aggregation in the colloidal solution. How to achieve this goal depends on the particular system. Among coacervating agents, electrolytes or amphiphilic counterions, as well as pH changes, are often used for inducing coacervation in colloidal solutions of ionic surfactants. In nonionic systems, one very effective way to promote coacervation is to lower the number of solvent molecules available for solvation, which is usually achieved by increasing the temperature. In the case of water insoluble nonionic surfactants, an excellent approach for their coacervation in organic solvents is using water as an inductor agent.[13]

Self-assembly of surfactants offers a unique opportunity to produce tailored nanostructured liquid phases able to improve yields, selectivity, sustainability and economics in extraction processes and become a viable alternative to organic solvents.[14] Thus, the nanostructures making up the coacervates have regions of different polarity that offer several types of interactions for solutes and consequently mixed mechanisms for their solubilization. As a result, solutes in a wide polarity range can be efficiently extracted. On the other hand, the high concentration of surfactant in coacervates (typically 0.1-1 mg/μL) makes them ideal platforms for amplification of solute binding. Thus, high extraction efficiencies can be achieved using low volumes of coacervates, which is important for extraction of contaminants present in aqueous solutions at very low concentration. One of the most interesting features of coacervates is that solvent properties can be tuned by proper selection of surfactants and the environment for their self-assembly. In this respect, coacervates featuring restricted access properties, which

able to extract low molecular weight solutes while excluding macromolecules, have been recently reported.[15]

So far, application of surfactant-based coacervates in extraction processes has invariably involved the use of single-chain surfactants.[13] Their applicability to pollutant removal in aqueous solution has been restricted by the fact that a substantial fraction of the surfactant is lost in the equilibrium solution where it is at the cac. So, removal of the surfactant in the equilibrium solution is mandatory after water treatment.[8]

In this work, we explore the coacervation of oligomeric surfactants as a strategy for reducing the cac in the solution in equilibrium with the coacervate. Lower cacs means that less surfactant is needed, which has both financial and environmental benefit, and that the scope of application of coacervates can be conveniently extended to the removal of pollutants in water purification and analysis.

Oligomeric surfactants are made up of two or more amphiphilic moieties covalently linked at the level of the hydrophilic head groups or at opposite ends of the hydrophobic chains giving intermediates namely of the head type and of the tail-end type.[16,17] Their cac decreases continuously with increasing the degree of oligomerization. Coacervation of gemini surfactants (i.e. two amphiphilic moieties connected by a spacer group at the level of the head groups) has been extensively reported[11,12] but, to the best of our knowledge, gemini-based coacervates have not been applied to the removal of pollutants in water.

Here, the coacervation of the oligomeric surfactant poly-undecylenic acid (Poly-UDA) in two hydro-organic media (i.e. tetrahydrofuran-water and ethanol-water) is investigated. Phase behavior and coacervate composition and volume, as well as the size of coacervate droplets as a function of the environmental conditions set for coacervation are studied. Oligomer concentration in the equilibrium solution is determined at the different conditions set for coacervation. Potential of the oligomeric coacervates for application to the removal of pollutants is discussed.

EXPERIMENTAL

Chemicals

Undecylenic acid, sodium persulfate, sodium hydroxide, and hydranal were purchased from Sigma-Aldrich (Steinheim, Germany). Tetrahydrofuran, ethanol, diethyl ether and hydrochloric acid of LC-MS grade were purchased from Panreac (Barcelona, Spain). Water (grade I) was obtained from an in-house purification system supplied by Merck (Darmstadt, Germany).

Synthesis and Characterization of Poly-undecylenic acid

Synthesis

The synthesis of poly-sodium undecylenate (Poly-SUD) has been previously reported by our research group.[18] Poly-undecylenic acid (Poly-UDA) was obtained by dissolving Poly-SUD in water and adding 5 equivalents of HCl_{cc}. The yellowish slurry obtained was then extracted with diethyl ether and finally isolated by evaporating to dryness. Figure 1.A shows a scheme of the synthesis of Poly-UDA.

Characterization

Purity of the product was checked by FT-MIR spectroscopy (Bruker Optik GmbH; Ettlingen; Germany). Furthermore, the molecular weight and polydispersity of Poly-UDA were measured using gel permeation chromatography, GPC (Viscotek, Malvern) and the particle mean diameter distribution, was determined by dynamic light scattering (Malvern Instruments, Malvern, UK). All assays were made in triplicate.

Coacervation of Poly-UDA

Phase behavior of the ternary mixtures Poly-UDA/Tetrahydrofuran/water and Poly-UDA/ethanol/water was investigated. A specific amount of Poly-UDA was weighted and solved by vigorously shaking in a known volume of organic solvent. Once the Poly-UDA was completely solved, water was added to the mix as coacervation-inducing agent. These mixtures were vortex-shaken and then centrifuged (14000g, 15

min) and they were observed to detect wether phase separation occurred. A general scheme of this process is shown in Figure 2.B.

Coacervate characterization

Chemical composition of coacervate was determined as follows: water was analyzed by coulometric Karl Fisher titration in hydranal. Poly-UDA was determined by weighing the solid obtained by ethyl ether extraction of the coacervate followed by evaporation to dryness; while the THF or ethanol content was calculated by difference. The volume of coacervate formed was measured using a digital calliper and calculating it using the height (h) and diameter (d) of the coacervate formed by the equation of a cylinder ($\pi * (d/2)^2 * h$). A non-linear regression model was built for the prediction of this volume as a function of the composition of the ternary mixture. Microphotographs of the coacervate synthetized were obtained by an optical microscopy Leica model (Barcelona, Spain) and by a cryo-scanning electron microscopy JEOL JSM-5410CT-1000 interfaced to a cryo-transfer system (Oxford Instruments, Oxford, UK).

RESULTS AND DISCUSSION

PolyUDA Synthesis and Characterization

The synthesis of poly-undecylenic acid (Poly-UDA), previously described in the experimental section, resulted in a yield of 90% in Poly-UDA, relative to the starting amount of Poly-SUD. The identity of the polymer was confirmed by studying the infrared spectrum of the solid obtained after polymerization. For this purpose, ATR infrared spectroscopy (attenuated total reflectance) was used. Infrared spectra obtained for the monomers (UDA) and the oligomeric product (Poly-UDA) are shown in Figure 2. In the spectrum of Poly-UDA, peaks characteristics of double bond stretching and bending vibrations (e.g., at 3080 cm^{-1}, 1630 cm^{-1}, 990 cm^{-1}, and 910 cm^{-1}) disappeared. This fact confirmed that there was no contamination from the monomer in the final product. A new peak at 1160 cm^{-1}, assigned to rigid methylene groups, suggested a successful polymerization.

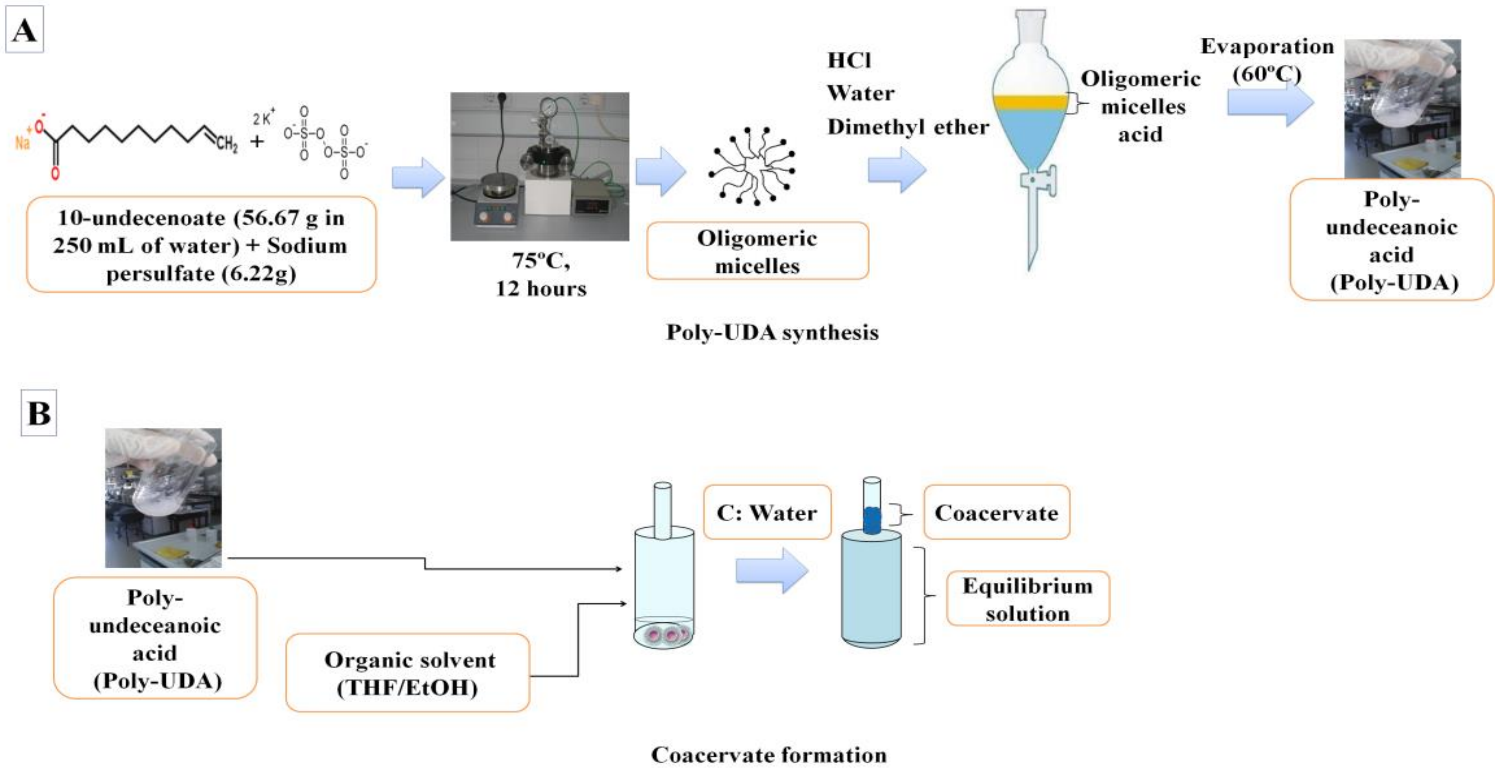

Figure 1. Scheme of Poly-UDA synthesis (A) and coacervate formation (B)

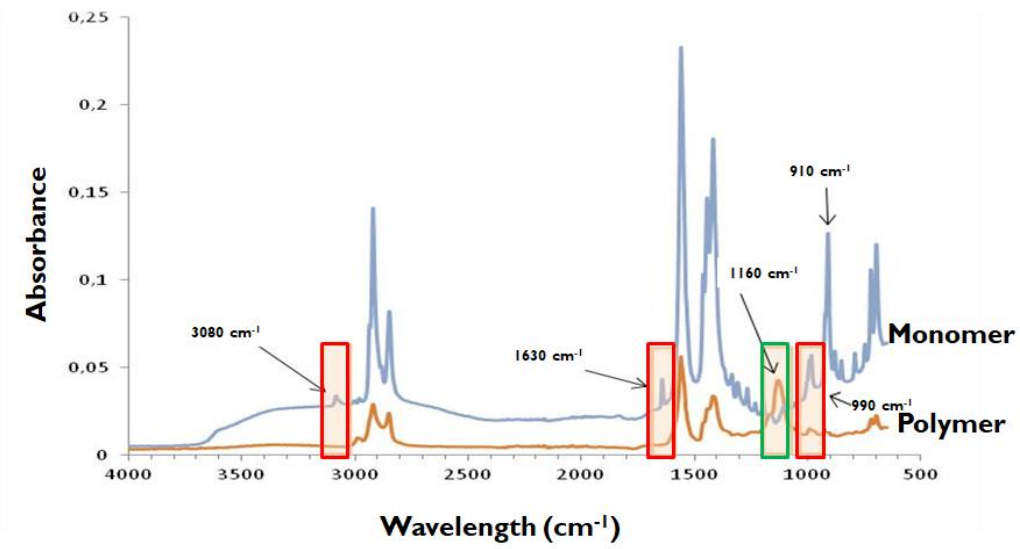

Figure 2. Infrared spectra of undecylenic acid (monomer) and poly-UDA (oligomer)

The GPC analysis of Poly-UDA (Figure 4) showed a single peak with a molecular mass of ~3300 ± 150 Da, which resulted in a degree of polymerization of 17 ± 1, that was in good agreement with previous results.[16] The oligomers arranged in micelles, with a micellar size, determined by DLS, of 3.1 ± 0.6 nm.

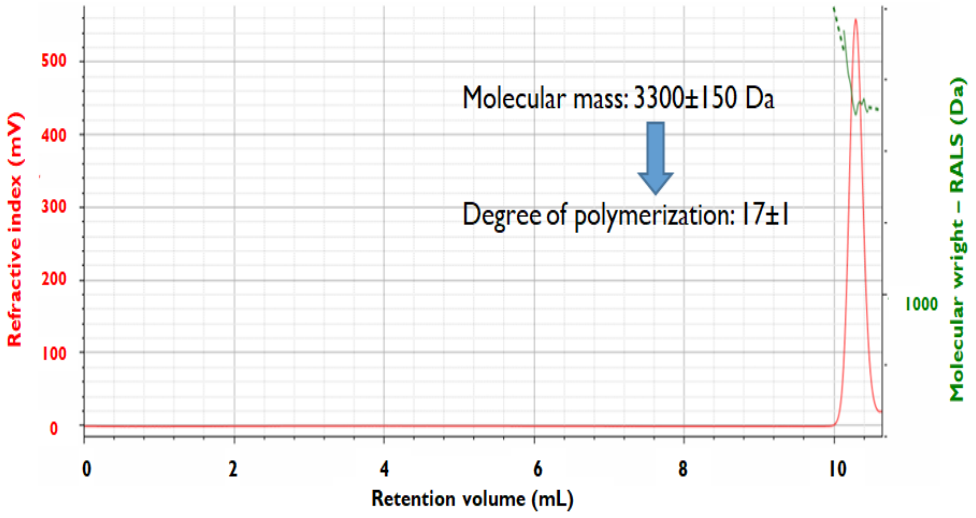

Figure 3. Gel Permeation Chromatogram of Poly-UDA

Phase Boundaries for Poly-UDA-Organic Solvent-Water Mixtures

Ternary phase diagrams (or Gibbs triangles) were built for ternary mixtures of Poly-UDA/tetrahydrofuran/water (Figure 4.A) and Poly-UDA/ethanol/water (Figure 4.B). Tetrahydrofuran and ethanol were selected as organic solvents because both are good solvents for Poly-UDA whereas their properties differ highly. Tetrahydrofuran is a moderately polar, water-soluble and aprotic solvent with a dielectric constant of 7.6. Meanwhile ethanol, a water miscible and protic solvent, being able to participate in hydrogen bonding and capable of dissolving ionic compounds, whose dielectric constant is 24.3.

Three well differentiated regions can be distinguished in both ternary diagrams. A precipitation zone, where a solid precipitate of Poly-UDA is obtained, an isotropic solution region where no aggregation or coacervation is observed, and a coacervate formation domain where a liquid-liquid phase separation is clearly obtained after centrifugation. In both diagrams, these domains correspond to the same synthetic conditions: an excess of water gives a precipitate and an excess of organic solvent result in a homogeneous solution.

However, it should be noted that the size of the coacervate formation region is quite different when comparing both ternary diagrams. This effect, that has also been demonstrated for coacervates based on alkyl carboxylic acids,[19] is related with the Hildebrand solubility parameter (δ), which measures the relative solvency behaviour of a solvent; reflecting the additive effect of dispersion, polar and hydrogen bonding forces. δ has a value of 5 for tetrahydrofuran and 15 for ethanol and therefore, the solute solvency ability for Poly-UDA is higher for tetrahydrofuran, which may explain the difference on size in the coacervate formation regions.

Additionally, the effect of temperature and salt concentration in the coacervation process were studied. It was found that concentrations of sodium chloride up to 1M did not result in any changes in the ternary phase diagrams previously obtained. In a similar way, rising the temperature of the synthetic mixture up to 75°C did not show any significant differences. This thermal and salinity stability assures a wider range of applicability for these new coacervates.

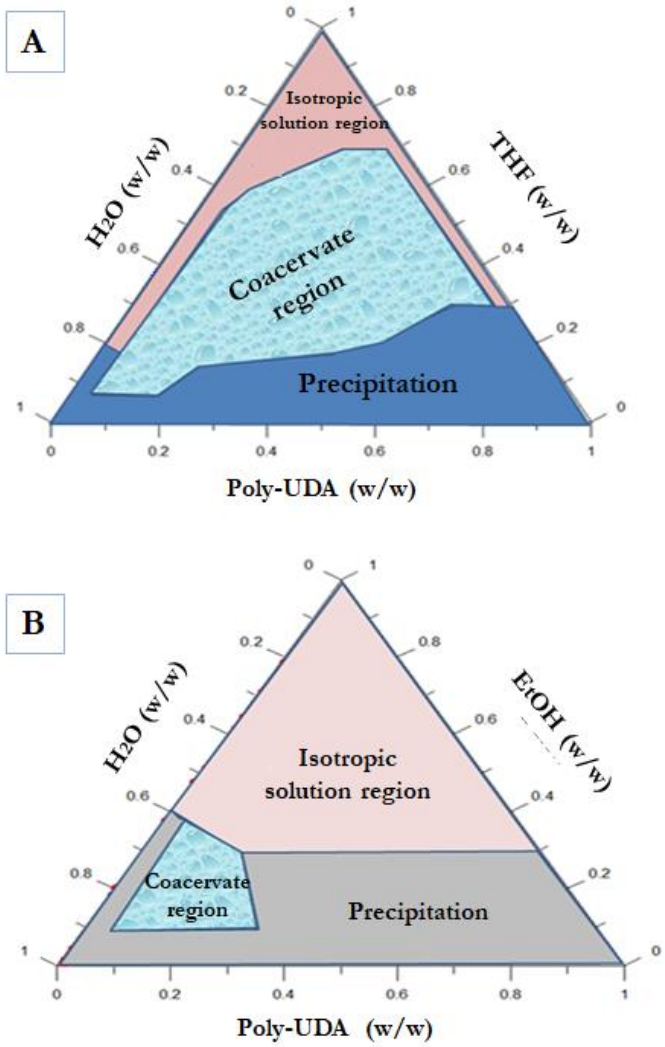

Figure 4. Ternary plots (w/w) for the mixtures Poly-UDA/THF/water (A) and Poly-UDA/ethanol/water (B)

Environment-Responsive Volume and Composition of Coacervates

As it has been previously stated for carboxylic acids, the composition of coacervates along their formation domain shows a high variability and, in that way, it can be tuned, in the sense that coacervates of different properties can be obtained just by starting the synthesis from slightly different environmental conditions. In this work, composition was studied for the coacervates formed by tetrahydrofuran and ethanol as organic solvents (Figure 5 and Table 1 and 2, respectively).

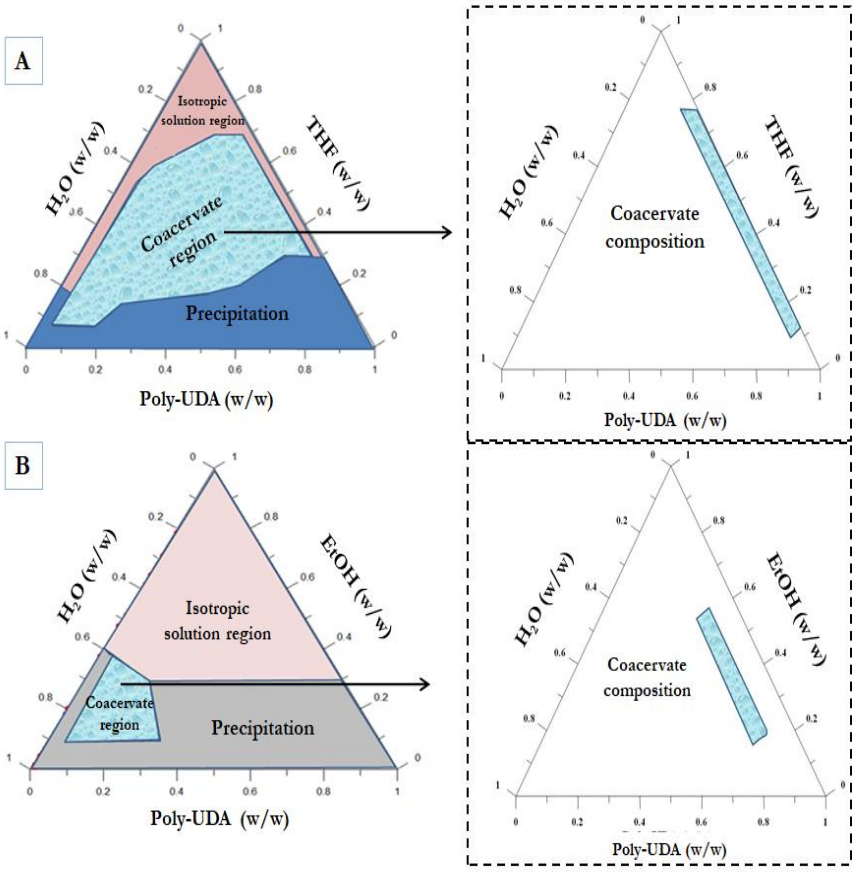

Figure 5. Ternary plot (w/w) for the Poly-UDA/THF/water synthetic mixture and Coacervate composition (inset) (A) and for the Poly-UDA/EtOH/water (B)

It should be highlighted that for all solvents and synthetic mixtures the incorporation of Poly-UDA to the corresponding coacervate is quantitative (>99%). This fact means a very big difference when comparing these new coacervates with previously synthetized and characterized ones, based mainly on carboxylic acids[19] and alkanols[15]. This hitherto unseen property clearly linked to the nature of oligomeric micelles, means that no amphiphile is lost into the equilibrium solution since this equilibrium for Poly-UDA, is totally displaced towards the coacervate.

Regarding organic solvent and water content in coacervates, they follow the usual trend found for those made up of monomeric carboxylic acids, with both, the organic solvent (i.e. THF or ethanol) and water, following a positive correlation with the organic solvent in the synthetic mixture. Therefore, coacervates with higher water content are situated in the upper part of the ternary phase diagram (Figure 5), where bulk solutions are formed by at least a 50% of organic solvent. It should be noted that the coacervates synthetized from ethanol shows higher proportions of water when compared with those synthetized from THF. This behaviour can be explained by the fact that ethanol, being a

protic solvent, shows a stronger interaction with water than that shown by tetrahydrofuran, an aprotic solvent. Finally, it is worth mentioning the high concentration of Poly-UDA in the coacervates, as a result of the complete incorporation of the oligomer from the bulk solution.

Table 1. Coacervate composition (w/w, %), as a function of the ternary synthetic mixture of Poly-UDA/THF/water

% Synthetic mixture (w/w)			% Coacervate composition (w/w)		
Poly-UDA	**THF**	**H_2O**	**Poly-UDA**	**THF**	**H_2O**
10	20	70	51.7	47.3	1.0
10	30	60	43.0	55.5	1.5
10	40	50	34.7	63.2	2.1
10	50	40	27.7	68.8	3.5
10	60	30	20.1	75.7	4.1
20	20	60	58.3	40.5	1.2
20	30	50	43.4	55.2	1.4
20	40	40	34.8	63.0	2.2
20	50	30	27.0	70.3	2.6
20	60	20	24.7	71.4	3.9
30	20	50	57.9	40.7	1.4
30	30	40	45.5	52.9	1.7
30	40	30	42.0	55.6	2.3
30	50	20	35.3	62.0	2.7
30	60	10	28.2	68.2	3.6
40	20	40	74.7	23.2	2.1
40	30	30	66.1	32.0	1.9
40	40	20	59.8	37.3	2.9
40	50	10	51.6	45.3	3.2
40	55	5	44.4	52.1	3.4
50	20	30	85.6	13.0	1.4
50	30	20	75.9	22.2	1.9
50	37	13	69.5	28.0	2.5

Table 2. Coacervate composition (w/w, %) as a function of the ternary synthetic mixture of Poly-UDA/ethanol/water

% Synthetic mixture (w/w)			% Coacervate composition (w/w)		
Poly-UDA	**EtOH**	**H_2O**	**Poly-UDA**	**EtOH**	**H_2O**
3	30	67	35.1	54.0	10.9
10	25	65	68.1	19.5	12.4
10	30	60	39.0	49.9	11.1
14	25	61	62.7	23.4	13.9
14	30	56	54.2	34.8	11.0
14	35	51	52.4	35.3	12.3

Another interesting parameter is the volume of solvent formed after coacervation. The prediction of this parameter is not only required to adjust the bulk solution to the desired amount of coacervate but also it is essential to predict the maximum concentration factor achievable in coacervate based extractions. In order to further study this parameter and to allow a fine tuning, a prediction equation was built linking the volume obtained to the synthetic mixture (i.e. amount of Poly-UDA, THF or ethanol, and water). Both equations (I and II) follow an exponential behaviour regarding organic solvent, which is in good agreement with the results previously obtained for coacervates based on alkanols[15] and alkyl carboxylic acids[19].

$$(I)V_{\text{coacervate}} = 384 + 844\ Ln(polyUDA)e^{0.023\ THF}$$

$$r^2: 0.96$$

$$(II)V_{\text{coacervate}} = 453 + 73\ (polyUDA)e^{0.011\ EtOH}$$

$$r^2: 0.96$$

where $V_{coacervate}$ is expressed in μL and Poly-UDA, THF and EtOH are the amount of these components in the bulk solution expressed as mmoles.

On the other hand, the usual behaviour of coacervates being linearly dependent on the amount of the surfactant was true for coacervates formed in ethanol (equation II) but nor for the one produced in THF (Equation I), which showed a logarithmic dependence. This different behaviour is related to the size of the coacervate formation region. Whilst previously studied coacervates and the new one based on ethanol are restricted to low proportions of amphiphile, the new coacervate based on THF reaches up to relative amounts of Poly-UDA higher than 90%. Under these conditions, a saturation in the linear dependence is shown, which results in a logarithmic behaviour.

The same experimental procedure was followed to predict the amount of water incorporated into the coacervate. The proportion by weight of water in the coacervate obtained from THF and EtOH showed different dependencies. For the one of THF, a dependence on the percentage (w/w) of water in the bulk solution and the solvent used was found, whilst for EtOH, water in the coacervate depended on the 3 components in the bulk solution

The derived equations were:

$$(a) W_{coacervate} = 4.99 + 0.11\, xTHF - 0.04\, xH_2O$$

r^2: 0.91

$$(b) W_{coacervate} = -406.72 + 4.88\, x\, EtOH + 1.45\, xH_2O + 300.48\, x\, polyUDA$$

r^2: 0.91

where $W_{coacervate}$ is the content of water in the coacervate expressed in g (a,b) and poly-UDA, THF and EtOH are the amount of these components in the bulk solution expressed in g of total solution.

The dependence of the solvent content of the SUPRAS on its content in the bulk solution was also described. The results obtained indicate that the solvent content depended on the ternary mixture. The derived equations for this study have been:

$$THF_{coacervate} = 7.26 - 0.66\, xTHF - 1.69\, xH_2O - 0.92\, x\, polyUDA$$

r^2: 0.84

$$EtOH_{coacervate} - 1694.4 + 429.5\, xEtOH + 338.9\, xH_2O + 339.52\, xpolyUDA$$

r^2: 0.84

where $THF_{coacervate}$ and $EtOH_{coacervate}$ are the content of organic solvent in the coacervate, expressed in g and Poly-UDA, THF and EtOH are the amount of these components in the bulk solution expressed as g.

Based on all these equations, it can be concluded that the spontaneous self-assembly process by which Poly-UDA forms coacervates follows predictable routes that can be used to obtain solvents with specific properties.

Environment-Responsive Size of SUPRAS Droplets

One of the main properties of coacervates is that they are micro-structured as an ordered cluster of independent droplets[19]. This was checked for the new SUPRAS studied by two microscopic techniques: light microscopy (Figure 6) and cryogenic

scanning electron microscopy (cryo-SEM) (figure 7). Light microscopy showed that droplet size was found to be dependent on the bulk solution composition; it increased as the proportion of organic solvent in the bulk solution did. In order to quantify this effect, mean diameters were inferred from microphotographies at different THF or ethanol proportions. Values measured were as follows: 30% THF, 50 μm; 40% THF, 100 μm; 50% THF, 200 μm; 25% ethanol, 40 μm; and 35% ethanol, 100 μm.

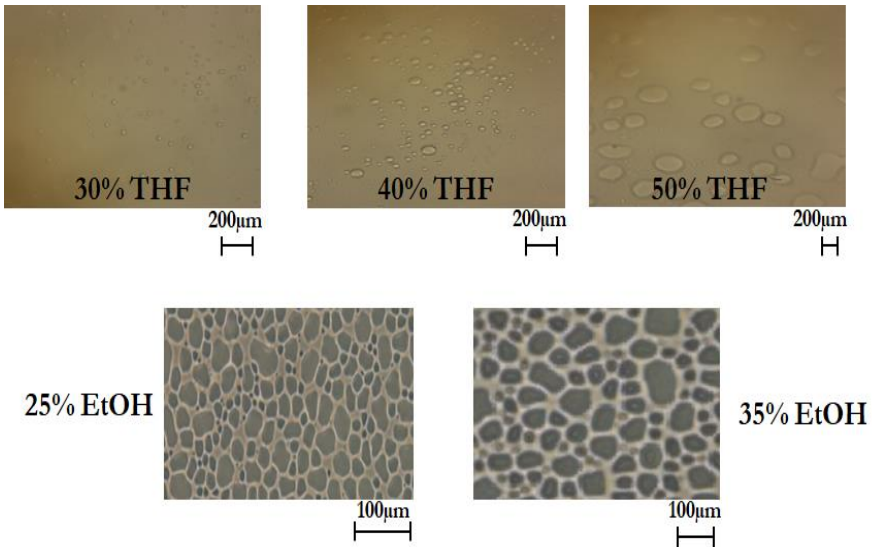

Figure 6. Light microphotographies obtained from coacervate at different THF and EtOH synthetic conditions

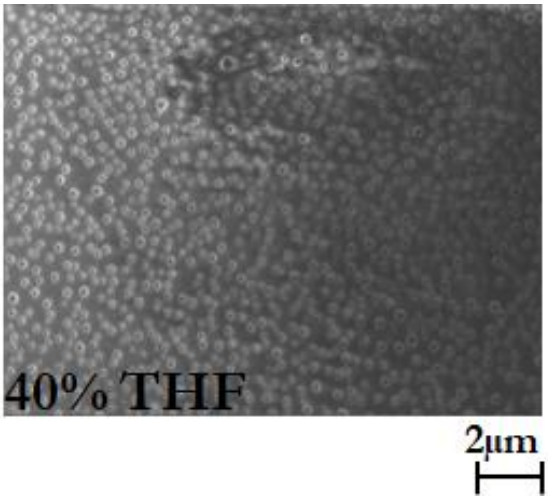

Figure 7. Cryo-SEM microphotographies obtained from coacervate at THF synthetic condition

Cryo-SEM, allowing higher magnifications than light microscopy, revealed the nature of the droplets, exposing that they are formed by nanodroplets with diameter the sub-micro domain (averaging ca.350nm). Keeping in mind the micellar size of a single unit of Poly-UDA (3.1nm), these nanodroplets formed by an assembly of molecules in

the range of 10^6, may represent a nano-scale organization, intermediate between single micelles and well-known SUPRAS main droplets. This result proves the fact that this novel SUPRAS is in fact an ordered liquid at the molecule-, nano- and sub- domains, as has been shown for other ones[15].

CONCLUSIONS

Coacervates based on an oligomeric surfactant made up of 17 monomeric units, a combination that had not been explored yet, shows some unique properties that may allow circumventing some of the limitations shown by these solvents. Firstly, it has been demonstrated that no traces of the oligomeric surfactant are lost into the equilibrium solution. This property is of special interest in fields where application of coacervates could be of great attraction, but the presence of surfactant may cause environmental and/or health issues, such as for example, water remediation or functional foods manufacturing. In our opinion, this new SUPRAS here synthetized and characterized offers a whole new set of features that need to be further explored and be taken full advantage of by researchers in a broad range of areas.

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Chapter III

High thermally stable supramolecular solvents applicable to headspace gas chromatography

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A HIGH THERMALLY STABLE OLIGOMER-BASED SUPRAMOLECULAR SOLVENT FOR UNIVERSAL HEADSPACE GAS CHROMATOGRAPHY: PROOF-OF-PRINCIPLE DETERMINATION OF RESIDUAL SOLVENTS IN DRUGS

José Ángel Salatti-Dorado, Soledad González-Rubio, Diego García-Gómez[1], Rafael Lucena, Soledad Cárdenas and Soledad Rubio

HIGHLIGHTS

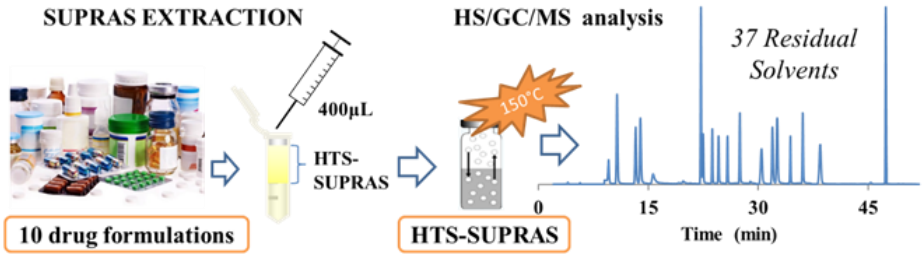
A supramolecular solvent with high thermal stability is described for the first time.

This new solvent is based on poly-undecylenic acid, an oligomeric surfactant.

It opens a new avenue for sample treatment prior to Headspace - Gas Chromatography.

A validated determination of 37 residual solvents in 10 different drugs was achieved.

GRAPHICAL ABSTRACT



KEYWORDS

Supramolecular solvent
Headspace gas chromatography
Residual solvents
Oligomeric amphiphiles
Pharmaceutical analysis

ABSTRACT

Supramolecular solvent (SUPRAS) extraction is gaining attraction as a sample treatment technique because of its great performance in terms of effectiveness, versatility, sample clean-up, quickness, cost and sustainability. However, the nature of SUPRASs, being formed by amphiphile molecules, results in poor compatibility with Gas Chromatography (GC). Here, we show the hither to unexplored development of a SUPRAS with high thermal stability (HTS) suitable for subsequent direct analysis by Headspace (HS)-GC. This novel HTS-SUPRAS, based on poly-undecylenic acid -an oligomeric surfactant-, tetraglyme -a polar aprotic solvent with excellent chemical and thermal stability- and water, was fully characterized in terms of composition and physical properties such as thermal stability. Subsequently, the HTS-SUPRAS developed was further successfully applied, as a proof-of-principle, to the extraction and determination of residual solvents in pharmaceutical drugs, including several solvents (class 2C) whose analysis by HS-GC has been shown to be highly complex. Analytical performance was demonstrated as mandated by the International Council for Harmonisation of technical requirements for pharmaceuticals for human use (ICH). Furthermore, excellent recoveries (70-120%) and high precisions (<20%, expressed as relative standard deviations) were obtained for the analysis of several different drug formulations spiked with the analyzed 37 residual solvents at their respective maximum residue levels.

INTRODUCTION

Supramolecular solvent (SUPRAS) extraction is a well-known analytical technique for sample extraction and clean-up [1]. Since its early introduction by Watanabe et al. as "cloud point technique" [2], SUPRAS extraction has evolved into a very attractive replacement to classical strategies [3]. Based on nano-structured liquids generated from

amphiphiles through a self-assembly process occurring at molecular scale and made up of large supramolecular aggregates dispersed in a continuous phase, SUPRASs show some unique properties (e.g. multiligand ability for solute binding) that render them very attractive for analytical extractions [1,3]. More recently, a further step to establish SUPRAS extraction as a broadly useful technique has been achieved by the development of functional SUPRASs as, for example, volatile SUPRASs (VOL-SUPRAS) [4] or SUPRASs with restricted access properties (RAM-SUPRAS) [5, 6]. Nevertheless, the main drawback when routinely applying SUPRAS extraction is the high concentration of amphiphiles (up to g mL^{-1}) in the extract that can hinder SUPRAS compatibility with mainstream analytical separation and detection systems [7]. This fact is of special important for Gas Chromatography (GC) since the presence of surfactants is highly detrimental to GC injectors and columns [8], and prevent analyte determination [1].

Different strategies have been developed to improve this compatibility, mainly based on the removal of the amphiphile by solid-phase extraction [9] or back-extraction [10]. An interesting strategy was also developed by Hinze et al. that, instead of removing the surfactant, is based on its derivatization [11]. Another approach may be the use of Headspace (HS) as a prior step to GC [12]. However, heating of the SUPRAS results in a high volume of organic vapors and different decomposition products that make the analysis impractical. In this sense, the development of a SUPRAS with high thermal stability (HTS-SUPRAS) would be of great interest since it may show an excellent compatibility with HS-GC, allowing the direct coupling of SUPRAS extraction and GC separation without any previous amphiphile removal or derivatization step. Thus, the excellent properties of SUPRAS extraction can be combined with the high separation power of GC to determine compounds in a wide range of polarity and volatility.

Residual solvents are often used during the synthesis of drugs to increase yield or assist crystallization [13]. Even though these solvents are critical to the synthesis, they have no therapeutic value and may show a potential risk for human health due to their toxicity and side effects [14]. Therefore, determination of residual solvents becomes a necessary procedure for quality control of drug substances and drug products to meet regulatory expectations and ensure patient safety. In this regard, the International Council for Harmonization of technical requirements for pharmaceuticals for human use (ICH) has developed a guideline [15] that recommends acceptable amounts for

residual solvents in pharmaceuticals to be adopted by the regulatory bodies of the European Union, Japan, USA, Canada and Switzerland.

Solvents are classified according to their toxicity into class 1 solvents (to be avoided), class 2 solvents (to be limited) and class 3 solvents (low toxic potential). Class 1 (5 solvents) and class 2 (32 solvents) are restricted to individual maximum concentration limits (see Table S-1 in the Supporting Information (SI)), with boiling points varying in a wide range of temperatures (32-285°C, Table S-1). Subsequently, different analytical methods, mainly based on HS-GC [16, 17], have been developed to assess concentration limits, including methods from official organizations (USP) [18]. Sample diluent is a critical factor in all these methods affecting sample load, sensitivity, and HS equilibration temperature and time. A good sample diluent for analysing residual solvents in drugs should show high capability for dissolving different samples, a high boiling point -HS equilibration temperature should be lower- and good stability.

There are several commonly used sample diluents such as water, DMSO, DMF, N,N-dimethylacetamide, benzyl alcohol, 1,3-dimethyl-2-imidazolidinone, and mixtures of water–DMF or water–DMSO. Some of these organic solvents may provide better solubilization and higher boiling points than water, however, they can be present in drugs as residual solvents themselves and they are not very stable at high temperatures and therefore susceptible to degradation during HS procedure. As a result, not enough vapor concentrations are achieved for residual solvents at the maximum temperatures reachable. Thus, a HS-GC method based on DMSO as sample diluent failed when applied to 10 out 44 solvents [16], because their boiling points were superior to that of the diluent. Likewise, the European Pharmacopea method [18] is not suitable for the determination of formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, N-methylpyrrolidone and sulfolane. Recently, ionic liquids have been reported as a suitable sample diluent for the analysis of high-boiling point residual solvents [19]. Ionic liquids have extremely low vapor pressures and can dissolve a wide range of compounds. However, when compared to other solvents, solution–vapor partition coefficients vary considerably for analytes dissolved in ionic liquids and, additionally, they have high purchase and purification costs [20].

On the grounds of these facts, it is the aim of this work to develop a SUPRAS with high thermal stability (HTS-SUPRAS) that may show direct compatibility as a universal solvent for HS-GC. As a proof-of-principle, it will be applied as a

comprehensive sample diluent for residual solvents in pharmaceuticals, including those of high boiling point (e.g. class 2C), surpassing in that way the existing analytical methods. For that purpose and as starting hypothesis, SUPRAS will be synthesized from oligomeric amphiphiles since they have been proved to show higher stability than monomeric amphiphiles [21-23], and glymes will be used as organic solvents since they offer high solvency, water solubility and a huge range of boiling points [24].

EXPERIMENTAL SECTION

Chemicals

All chemicals were of analytical-reagent grade, ≥99.9% purity if the contrary is not stated and employed as supplied. LC grade methanol and acetonitrile were supplied by VWR Chemicals (Llinars del Vallés, Barcelona). 10-undecylenic acid (UDA, ≥96%), decanoic acid (≥98%), potassium persulfate (≥99%) and Tetraethyleneglycol dimethyl ether (Tetraglyme, ≥99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (37%), tetrahydrofuran, diethyl ether and ethanol were supplied by Panreac (Castellar del Vallès, Spain) and sodium hydroxide (NaOH) by Merck (Darmstadt, Germany). Ultra-pure water was produced in a Millipore (Billerica, MA, USA) purification system. Standard mixtures of Residual Solvents for Class 1 (at 5000x their concentration limits) and Class 2A, 2B and 2C (at 5x their concentration limits) were purchased from Restek (Benner Circle, Bellefonte, PA, USA). Working solutions were prepared daily by diluting the proper amount of these mixtures.

Synthesis of Poly-Undecylenic Acid (Poly-UDA)

The synthesis of sodium poly-sodium undecylenate (Poly-SUD) has been previously reported by our research group [25]. Poly-undecylenic acid (Poly-UDA) was obtained by dissolving Poly-SUD in water and adding 5 equivalents of HCl_{cc}. The yellowish slurry obtained was then extracted with diethyl ether and finally isolated by evaporating to dryness. A yield of ca. 90% in Poly-UDA, relative to the starting amount of Poly-SUD, was obtained.

Synthesis and Characterization of Oligomer-based SUPRAS

The synthesis of oligomeric-based SUPRAS was carried out by dissolving 2.0 g of Poly-UDA into 4.0 mL of tetraglyme in a centrifuge tube and then adding 4.0 mL of water. This solution was subjected to vortex stirring (3000 rpm) for 1 min. The SUPRAS formed spontaneously by self-assembly and coacervation. It was separated from the equilibrium solution by centrifugation at 14250g for 10 min and then it was transferred to a vial and stored at room temperature.

Different physicochemical properties of the SUPRAS were determined. Thus, the volumes of SUPRAS obtained under different initial conditions were measured by means of a digital caliper from Medid Precision, S.A. (Barcelona, Spain). Water content in the SUPRAS was determined by means of a Coulometric Karl Fischer titrator (Metrohm, Herisaus, Switzerland), while the poly-UDA content was calculated by measuring its remnant in the equilibrium solution after SUPRAS formation and separation. For this purpose, Poly-UDA remaining in the equilibrium solution was extracted with diethyl ether, followed by vacuum evaporation to dryness of the extract and weighing of the solid residue. The content of tetraglyme in the SUPRAS was calculated by difference. Thermal stability of the SUPRAS was determined by thermogravimetric analysis (TGA) with a Mettler Toledo TGA/DSC-1 working at a heating rate of 5°C min^{-1} from 25 to 600 °C under nitrogen or oxygen atmospheres (100 mL min^{-1}).

Determination of Residual Solvents in Drugs

Samples

Ten pharmaceutical formulations were bought at local drugstores. The samples involved different active pharmaceutical ingredients and excipients (Table S-2 in SI) and solid and liquid formulations. They were stored as instructed by suppliers.

SUPRAS-Based Extraction of Residual Solvents

Two different experimental procedures were followed depending on the nature of the drug formulation. For solid drugs (e.g. tablets, capsules, etc.), 200 mg of sample

were diluted in 400 mg of HTS-SUPRAS (1:2 ratio of sample diluent). After homogenization by vortex-shaking (2 min at 3000 rpm), this solution was transferred to a glass vial and subsequently analyzed by HS-GC-MS. For liquid formulations (i.e. emulsions and injections), sample dilution is not advisable because of their high-water content that may cause overpressures in the HS system. For these samples, residual solvents were extracted by SUPRAS synthesized *in situ* in the sample. For this purpose, 330 mg of Poly-UDA were dissolved into 665 μL of tetraglyme in a centrifuge tube and, afterwards, 465 μL of water and 200 μL of aqueous pharmaceutical sample were added. After vortex-shaking (2 min at 3000 rpm), spontaneous coacervation, and centrifugation (14250g for 10 min at 10ºC), the 400 mg of HTS-SUPRAS formed were transferred to a glass vial, which was immediately sealed and later analyzed by HS-GC-MS.

Headspace-Gas Chromatography-Mass Spectrometry

HS-GC-MS analyses were run in an Agilent 6890N (Agilent Technologies, USA) equipped with a quadrupole mass spectrometer detector operated in the EI mode (70 eV), and a split/splitless injector. Compounds were separated on a DB-624 (30 m x 0.25 mm x 1.4 μm) capillary column formed by 3.5% Cyanopropyl, 3.5% Phenyl and 93% Methyl polysiloxane (Agilent). Helium was used as carrier gas (1.0 mL min^{-1}). The MS was set in scan mode (m/z 30 to 140) with a solvent delay of 1 min. The injector was operated in split mode (1:10) at 150°C. The separation of the 37 residual solvents was conducted with a temperature programming as follows: 35°C hold for 2 min, an increase to 65°C at 1.5°C min^{-1} hold for 10 min and finally and increase to 260°C at 10°C min^{-1}. Headspace sample generation was performed incubating the vials at 150°C for 45 min under a continuous mechanical stirring at 500 rpm in the autosampler (Gerstel, Germany). The syringe (2.5 mL) was set at 150°C and 500 μL of sample were injected. The total chromatographic run time was 70 min. Analytes were identified by comparison to the NIST database (NIST/EPA/NIH Mass Spectral Library, v. 2.0). For quantification, chromatographic peaks were extracted for the main ion for each residual solvent.

Method Validation

The HS-GC-MS method based on HTS-SUPRAS was validated as a "quantitative test for impurities" according to the *ICH Guideline for Residual Solvents Q3C (R6)* [15] that refers to the *ICH guidelines Text on Validation of Analytical Procedures* [26], evaluating the following parameters: accuracy, by assessing recoveries from samples spiked with known amounts of impurities; precision as repeatability (3 concentrations/3 replicates) and intermediate precision (interday); specificity by analyzing several spiked and unspiked pharmaceutical formulations; detection and quantitation limits based on the standard deviation of the response (residual standard deviations of regression lines) and linearity range, by a linear regression line based on 6 concentration levels (maximum concentration tested 250% of the concentration limit reported in Table S-1 in SI). Stability was assured by monitoring the storage conditions of the analytes and controlling the conservation parameters of the samples as recommended by the supplier.

RESULTS AND DISCUSSION

Synthesis and Characterization of Oligomer-Based SUPRAS

One of the most versatile process to induce coacervation (i.e. synthesize a SUPRAS), as has been shown in the last years [1, 3], is the addition of a poor solvent for the amphiphile that is miscible with the solvent used for its previous solubilization. Competition for this solvent will desolvate surfactant polar groups, thus causing micellar growth and phase separation. In this sense, water-induced SUPRASs [5, 27], which are the most studied SUPRASs, exhibit excellent properties such as tunability, multiligand ability and even the capability of acting as restricted access materials [5, 6]. The synthetic procedure involves the solution of the surfactant, usually a carboxylic acid or and alkanol, into a water-miscible organic solvent -tetrahydrofuran being the most used- and the addition of the proper amount of water to induce coacervation. It is obvious that for obtaining an HTS-SUPRAS two components of the procedure needs to be improved: a high boiling point water-miscible organic solvent instead of tetrahydrofuran and an amphiphile with higher thermal stability.

Regarding the organic solvent, glymes may be a viable alternative. These high-performance solvents [28] are aprotic, saturated polyethers that offer high solvency, high stability and a wide range of solubilities and boiling points. They are used as reaction solvents and in closed loop applications such as gas scrubbing and in refrigeration systems, among many other applications [24]. From the different glymes available, tetraglyme was selected for this work mainly for offering high water solubility, a boiling point of 276°C and a vapor pressure below 0.01 hPa (20°C) [28]. As concerns the amphiphile, a higher thermal stability can be obtained by using oligomeric surfactants. These compounds are a family of polymeric amphiphiles whose synthesis and characterization has long been reported [21-23]. For polyundecylenic acid (Poly-UDA), one of the most studied oligomeric surfactants, it has been demonstrated that aqueous micelles are formed at all concentrations, i.e. there is no critical micelle concentration (cmc) [21]. It should be noted that these lasting micelles show high stability because there are no single monomers to be exchanged with each other. Consequently, Poly-UDA was selected as a promising amphiphile for the synthesis of the HTS-SUPRAS. Poly-UDA, as stated in the experimental section, was arranged from Poly-SUD previously obtained by a synthesis developed in our group [25], which yields Poly-SUD of 3300±150 Da (degree of polymerization of 17±1) with a micellar size of 3.1±0.6 nm.

SUPRASs produced in hydro-organic media are environmental responsive and, consequently, their main properties depend on the synthetic initial conditions [1, 3]. Therefore, to fully characterize the HTS-SUPRASs developed in this work, different mixtures of Poly-UDA, tetraglyme and water were tested. Results are summarized in a ternary plot (Figure 1) where three different domains can be distinguished: an isotropic solution zone where water content was not enough to induce coacervation; a precipitation zone, with not enough tetraglyme to keep Poly-UDA in solution; and a SUPRAS formation domain. This behavior was in good agreement with that found for water-induced SUPRAS formed by monomeric alkanols or carboxylic acids and tetrahydrofuran [5, 27].

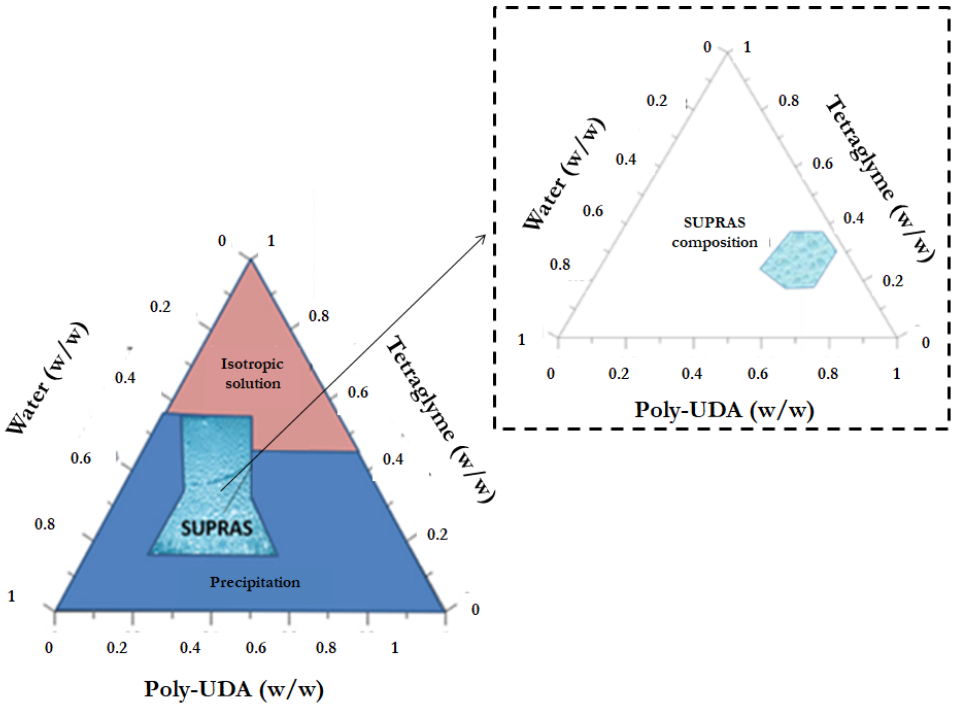


Figure 1. Ternary plot (w/w) for the Poly-UDA/tetraglyme/water synthetic mixture and HTS-SUPRAS composition (inset)

The SUPRAS formation region was further characterized by analyzing the chemical composition of the liquid phases obtained from nine different synthetic mixtures (Table 1). Firstly, it was checked whether Poly-UDA was fully incorporated into the SUPRAS, as it was expected from the fact that there is not cmc and, therefore, there is no equilibrium with monomers, or there was some Poly-UDA remaining in the equilibrium solution. A complete incorporation was found for all the synthetic mixtures tested (data not shown). Secondly, the environment-dependency of HTS-SUPRASs was checked, which resulted in SUPRAS that can be changed at will by selecting the adequate proportion of Poly-UDA, tetraglyme and water in the synthetic solution. Thus, SUPRASs made up of a constant percentage of Poly-UDA and increasing percentages of tetraglyme can be easily designed and obtained starting from the opposite conditions in the synthetic solution, viz., from increasing and constant percentages of Poly-UDA and tetraglyme, respectively (e.g. see conditions 1-3, 4-5, 6-7 and 8-9 in Table 1). This behavior derives from the complete incorporation of Poly-UDA from the synthetic solution into the HTS-SUPRAS. In this sense, the higher the Poly-UDA concentration, the higher the concentration of tetraglyme needed in the SUPRAS for its solubilization. This behavior opens the door to the synthesis of HTS-SUPRAS in which water composition can be modulated.

Table 1. Volume and Chemical Composition for HTS-SUPRAS Obtained from Different Synthetic Mixtures

SUPRAS Formation conditions	**Synthetic mixture/% weight**			**SUPRAS volume/μL g^{-1} of initial mix**	**SUPRAS composition/% weight**		
	Poly-UDA	**Tetraglyme**	**water**		**Poly-UDA**	**Tetraglyme**	**water**
1	20	20	60	220	62.5	22.5	15
2	30	20	50	330	62.5	30	7.5
3	40	20	40	440	62.5	32.5	5
4	20	30	50	230	60	20	20
5	30	30	40	340	60	35	5
6	20	40	40	240	55	30	15
7	30	40	30	380	55	35	10
8	10	50	40	130	50	25	25
9	20	50	30	260	50	30	20

Figure 1 (inset) shows the ternary phase diagram obtained for the composition of SUPRASs synthesized from different experimental conditions. It can be easily inferred that SUPRASs feature high amphiphile content, compared to that of water and, subsequently, they offer a high number of sites for solute solubilization.

The volume of HTS-SUPRAS obtained per gram of synthetic mixture (Table 1) was linearly dependent on the relative amount of poly-UDA in the synthetic solution and exponentially dependent on tetraglyme (equation 1), in a similar way that SUPRAS made up of monomeric amphiphiles in hydro-organic media [5, 27].

$$V_{SUPRAS} = (9.8 \pm 0.2)\ \text{polyUDA}\ e^{(5.9\pm0.6)10^{-3}\text{tetraglyme}}$$

where concentrations of Poly-UDA and tetraglyme refer to percentages in weight (w/w) in the synthetic solution. This equation allows, under any given conditions, the prediction of the volume of the HTS-SUPRAS generated and, therefore, the volume of diluent available and the calculation of the corresponding preconcentration factors for liquid-liquid extraction applications in which SUPRAS is synthetized *in situ*.

In order to check the suitability of HTS-SUPRASs for HS-GC, they were subjected to thermal gravimetric analysis (TGA). Figure 2 shows, as an example, the TGA thermal curves obtained in nitrogen (Fig. 2A) and oxygen (Fig. 2B) atmospheres for an HTS-SUPRAS synthesized from Poly-UDA, tetraglyme and water at the proportions 20/40/40 (w/w/w). This SUPRAS (number 6 in Table 1) contains an intermediate percentage of water (15%). For comparison purposes, the TGA thermal curves obtained for a SUPRAS made up of a monomeric surfactant (decanoic acid) in THF-water were also included in Figure 2.

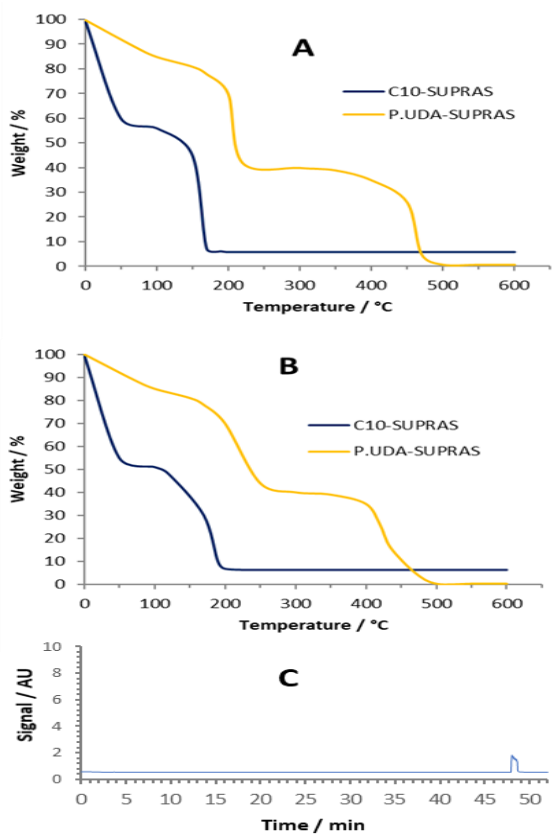


Figure 2. Thermogravimetric analysis (TGA) of the HTS-SUPRAS and a decanoic acid (C10) SUPRAS under nitrogen (A) and oxygen (B) atmospheres. Blank HS-GC-MS chromatogram obtained from an HTS-SUPRAS (C)

As it can be seen, at 150ºC, only around a 15% of the HTS-SUPRAS weight is lost, accurately corresponding to its water composition. Even though this loss of water showed to be compatible with HS-GC, it should be noted that it could be minimized if necessary by tuning the water composition of the HTS-SUPRAS to values as low as 5% (Table 1). No further decomposition from the amphiphile or the solvent was shown up to 200ºC. Beyond this temperature and up to 250ºC, a high loss of ca. 40% is shown, which may correspond to the complete loss of tetraglyme (30%) and a partial decomposition of the oligomeric amphiphile, as was confirmed by TGA analysis of the Poly-UDA itself (data not shown). The remaining 40% of weight was removed from 400ºC to 500ºC, yielding a negligible residue over this temperature. On the other hand, the decanoic acid SUPRAS lost a 50% of its mass at ca. 50ºC and only a small amount of residue (~5%) was left at 200ºC. No significant differences were found under nitrogen or oxygen atmospheres.

The excellent thermal stability of SUPRASs produced from oligomeric surfactants in glymes-water media makes them a promising universal diluent media for HS-GC. Figure 2C shows a blank chromatogram, obtained as stated in the experimental section, from an HTS-SUPRAS. It should be noted that no significant interferences were found in the background profile, being the baseline chromatogram similar to that obtained by using ionic liquids as diluent solvent (for the single peak at 48.5 min, see below), and much less noisy than those from high boiling point solvents such as N-methylpyrrolidone [19].

Method Optimization

In order to check the applicability of the HTS-SUPRAS in HS-GC-MS, we focused on the development of an analytical method for the determination of residual solvents that could be applied to test pharmaceutical drugs as proof-of-principle. Separation of the 37 solvents (classes 1 and 2) was achieved under the HS-GC-MS conditions stated in the experimental section, similar to those described in the US Pharmacopeia [18]. It should be highlighted that headspace temperature was set at 150°C, necessary to reach enough vapor concentration for high boiling point compounds. Such a high temperature cannot be applied in existing methods -involving 80 to 105°C- [[16], [17], [18]] that, subsequently, fail to properly determinate several class residual solvents. This temperature was selected as optimum since it resulted in excellent analytical characteristics (see below) whilst not lengthening analysis time and not forcing GC instrumental parameters (e.g. syringe temperature). Nonetheless, as shown above, the HTS-SUPRAS may allow even higher temperatures to be reached if necessary.

From the different HTS-SUPRAS available (Table 1), the one formed by an initial mixture of 20/40/40 (Poly-UDA/tetraglyme/water) was selected for further analyses. This selection was based on the mean properties of this SUPRAS (e.g. 15% of water composition), which make it a good representative, and on the fact that it lays on the middle of the SUPRAS region formation, which means that any changes in uncontrolled parameters (e.g. temperature) do not result in precipitation of total solubilization, i.e., selecting this particular HTS-SUPRAS yields a more robust synthesis process. Initially, a blank HTS-SUPRAS sample was run to check for possible overpressures and interferents arising from the HTS-SUPRAS itself. As expected, no overpressure

problems were detected, and a baseline chromatogram was obtained (Figure 2C). A single chromatographic peak was observed at 48.5 min that was confirmed as tetraglyme coming from the HTS-SUPRAS (Figure S-1). Keeping in mind that tetraglyme concentration is constant through all samples, it was used in subsequent analyses as internal standard to improve precision.

The HS-GC-MS extracted ion chromatograms obtained from an HTS-SUPRAS spiked with the 37 residual solvents at their respective concentration limits is shown in Figure 3 (Class 2C) and in the Supporting Information (Figure S-1, rest of classes). It should be noted that the presence in the SUPRAS of different polarity regions provides excellent solvation properties for a variety of organic and inorganic compounds, which means that a very high drug formulation/SUPRAS solubility ratio could be easily achieved for all drugs, despite their different nature (Table S-2). This ratio was maximized, to optimize analytical limits as much as possible, by studying values in the range 1/10-1/1 (drug formulation/SUPRAS). It was found that ratios from 1/10 to 1/2 resulted in similar chromatograms with the corresponding improvement in signal-to-noise ratios. However, results for 1/1 did not follow this trend. A closer examination showed that, for some formulations, a total drug solubilization was not achieved for a 1/1 ratio. Therefore, 1/2 was selected as the optimum solubilization ratio that, when compared with ratios proposed by other methods (e.g., USP 1/120), highlights the great performance shown by the HTS-SUPRAS in terms of solubility. For that reason, we suggest that, thanks to HTS-SUPRAS specific properties, easier, quicker and more sensitive methods for sample treatment prior to HS-GC could be established.

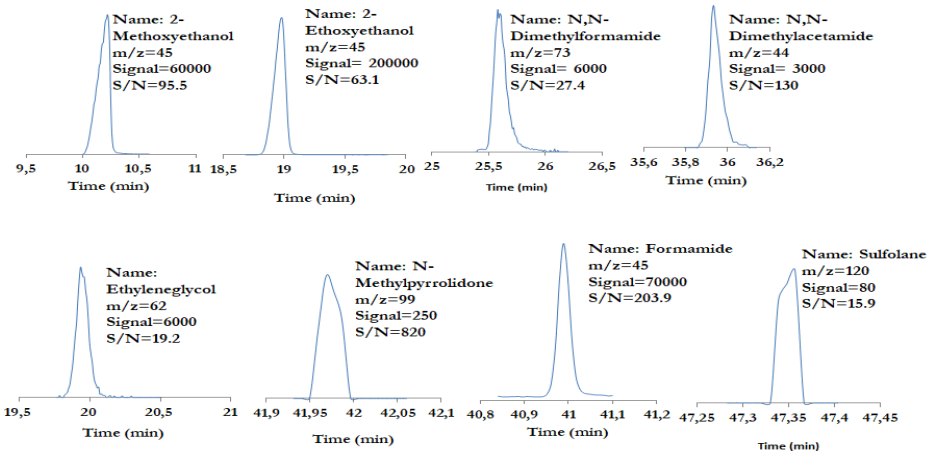


Figure 3. Class 2C HS-GC-MS extracted ion chromatograms obtained from an HTS-SUPRAS spiked with the 37 residual solvents at their respective concentration limits

Method Validation

Afterwards, the analytical method developed was fully validated according to ICH guidelines as a quantitative test for impurities [26]. Initially, satisfactory calibration curves were built for the 37 analytes in the range from their quantitation limits to 250% their respective concentration limits (Table 2 for Class 2C and Table S-3 for the rest of classes). Two analytical limits were determined: the detection limit and the quantification limit (Tables 2 and Table S-3). Values were, for all analytes, well below the legislated concentration limits (CL), even for the solvents with the highest boiling points. Precision -as repeatability and intermediate precision (interday)- was determined by spiking HTS-SUPRASs with each residual solvent at 50%, 100% and 150% their respective CLs. The relative standard deviations are shown in Tables 2and Table S-3. All values were lower than 20%. Accuracy values, at the same three concentration levels, were also calculated as recoveries by spiking HTS-SUPRAS not previously used for calibration. Satisfactory values in the range from 85% to 120% were found (Tables 2 and Table S-3). A comparison with previous analytical methods shows that HS-GC-MS based on HTS-SUPRAS is superior to classical methodologies based on high-boiling point solvents. Whilst analytical performance is akin, with limits of detection well below legislated residue limits and satisfactory accuracies and precisions, this novel method can determine all the solvents included in the ICH guideline [15], with methods depending on DMSO [16] an even those established by the European Pharmacopea [18] failing when applied to 10 out of 44 and 6 out of 37 solvents, respectively. Of special interest is the comparison of the method here proposed with that depending on ionic liquids [19]. Both methods yield a baseline chromatogram, which circumvents the main disadvantages shown by classical approaches. Regarding analytical performance, limits of detection on the same order of magnitude -low ppm range-are achieved by both methodologies, as well as satisfactory recoveries from real samples. On the other hand, the number of formulations (3) and solvents (18, not including any class 2C solvent), studied by the method based on ionic liquids, are far too scarce to establish a proper comparison. Furthermore, it should be highlighted that ionic liquids have a cost of $2.25 per gram [19], fifteen times higher than solvents as DMSO or N-methylpyrrolidone ($0.15/g). Nonetheless, the cost of the HTS-SUPRAS proposed has been calculated to be around $0.30 per gram, only twice the price of classical solvents.

Table 2. Validation parameters for the Class 2C residual solvents obtained by the proposed HS-GC-MS method based on HTS-SUPRAS

Class 2C solvent	Retention time /min	Linear range / %CL	DL / %CL	Repeatability (Intermed. precision) / %			Recovery / %		
				50% CL	100%CL	150% CL	50% CL	100% CL	150% CL
2-Methoxyethanol	10.5	15-250	5.0	9 (18)	8 (14)	7 (11)	108±7	97±7	98±10
2-Ethoxyethanol	19.0	16-250	5.5	12 (14)	12 (20)	10 (15)	106±7	96±9	98±15
Ethyleneglycol	20.7	22-250	7.5	13 (20)	7 (18)	6 (14)	107±12	94±14	102±13
N,N-Dimethylformamide	26.1	22-250	7.5	11 (15)	12 (13)	8 (15)	103±14	100±10	96±11
N,N-Dimethylacetamide	35.9	12-250	4.0	10 (15)	11 (18)	8 (13)	105±12	97±15	100±9
Formamide	41.0	28-250	9.5	9 (18)	13 (19)	6 (14)	109±11	101±15	94±15
N-Methylpyrrolidone	42.0	42-250	14	12 (20)	12 (16)	10 (13)	118±9	96±9	91±8
Sulfolane	47.3	26-250	8.5	12 (14)	11 (15)	9 (12)	91±13	105±10	102±10

Analysis of Residual Solvents in Pharmaceutical Formulations

In order to validate the applicability of the HS-GC-MS method developed, based on HTS-SUPRAS, ten different pharmaceutical drugs (Table S-2), involving different pharmaceutical formulations, were analyzed as stated in the experimental section. None of the samples contained any residual solvent above their concentration limits. However, in spite of the fact that no non-compliant drug was found, there was a sample that did contain residual solvents above the quantification limits of the method. Table 3 shows the residual solvents found and their respective concentrations and Figure S-2 the corresponding extracted ion chromatograms.

Table 3. Residual solvents determined from an unspiked drug (suspension in Table S-2) analyzed by the proposed method based on HTS-SUPRAS (n=6)

Residual Solvent	Class	Concentration / ng mL^{-1}	CL / ng mL^{-1}
Dichloromethane	2A	240±20	600
1,4-dioxane	2A	160±20	380
Cumene	2A	22±2	70
Hexane	2B	200±20	290

Afterwards, the ten samples were spiked with each solvent at their respective concentration limits. Depending on drug formulation, drug samples were then solved in the SUPRAS in a 1/2 ratio, as stated in the experimental section for solid samples (Table S-2) or extracted by HTS-SUPRAS, as it is the case of aqueous liquid samples, i.e. injections and emulsion (Table S-2). Even though validation studies usually require the analysis of certified reference materials (CRM), it is also possible to substitute these materials by calculating recoveries, defined as the real concentration of a substance recovered along the whole analytical procedure, when CRMs are not available, as it is. The results obtained are shown in Figure 4 (Class 2C) and in Figure S-3 (rest of classes). It should be noted that the recoveries, for all 10 matrices -including several different solid and liquid formulations- and 37 analytes, were in the usually accepted range of 70%-120%. This fact highlights the broad applicability, accuracy, specificity and robustness of the method that did not show any significant matrix effects.

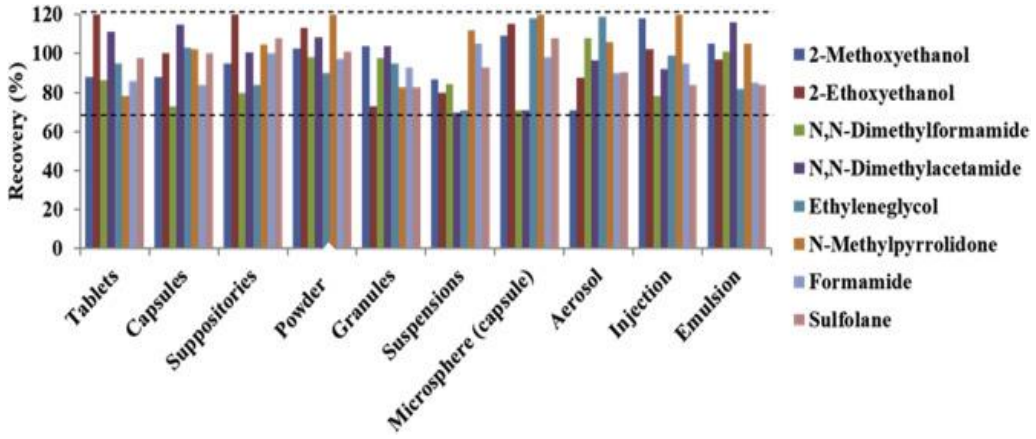


Figure 4. Recoveries obtained for class 2C residual solvents by the analysis of ten different drugs

CONCLUSIONS

For the first time, a novel SUPRAS with high thermal stability is proposed. This HTS-SUPRAS, based on an oligomeric surfactant, still keeps all the properties and functions shown by SUPRASs but, at the same time, allows to surpass some of the inherent disadvantages of SUPRAS formed by volatile components. In this sense, the poor compatibility of SUPRASs with GC hindered several analytical applications that, as it has been shown in this work for the case of residual solvents in drugs as proof-of-principle, can now be approached via the unique HTS-SUPRAS developed. When compared to alternative solutions, such as high boiling point solvents or ionic liquids, HTS-SUPRAS has been shown to offer a better behavior in terms of solubility, thermal stability, analytical performance, ease of synthesis and/or cost. It is our opinion that this HTS-SUPRAS opens a new avenue for sample treatment prior to HS-GC and that many applications may arise in the near future. In addition, further applications beyond analytical chemistry, where a non-volatile SUPRAS may be useful, should be explored.

ACKNOWLEDGEMENTS

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APPENDIX A. SUPPLEMENTARY DATA

The following is the Supplementary data to this article:

SUPPORTING INFORMATION

A HIGH THERMALLY STABLE OLIGOMER-BASED SUPRAMOLECULAR SOLVENT FOR UNIVERSAL HEADSPACE GAS CHROMATOGRAPHY: PROOF-OF-PRINCIPLE DETERMINATION OF RESIDUAL SOLVENTS IN DRUGS

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ADDITIONAL TABLES

ADDITIONAL FIGURES

Table S-1. List of residual solvents classified as class 1 and 2 according to the international council for harmonization of technical requirements for pharmaceuticals for human use (ICH) and their respective concentration limits and boiling points

Class	Residual Solvent	Concentration limits / mg L^{-1}	Boiling points / °C
1	1,1-Dichloroethene	8	32
	Carbon tetrachloride	4	77
	1,1,1-Trichloroethane	1500	74
	Benzene	2	80
	1,2-Dichloroethane	5	84
2A	Methanol	3000	65
	Acetonitrile	410	82
	Dichloromethane	600	40
	1,2-dichloroethene	1870 (50% Z, 50% E)	60/48
	Tetrahydrofuran	720	66
	Cyclohexane	3880	81
	Methylcyclohexane	1180	101
	1,4-Dioxane	380	101
	Toluene	890	111
	Chlorobenzene	360	131
	Xylene	2170 (60% meta, 14% para, 9% orto, 17% ethyl)	138/139/144/136
	Cumene	70	153
2B	Hexane	290	69
	Chloroform	60	61
	Nitromethane	50	101
	1,2-Dimethoxyethane	100	85
	1,1,2-Trichloroethene	80	87
	Pyridine	200	115
	Methylisobutylketone	4500	117
	Methylbutylketone	50	128
	Tetralin	100	207
2C	2-Methoxyethanol	50	124
	2-Ethoxyethanol	160	136
	Ethyleneglycol	620	197
	N,N-Dimethylformamide	880	153
	N,N-Dimethylacetamide	1090	165
	N-Methylpyrrolidone	530	210
	Formamide	220	202
	Sulfolane	160	285

Table S-2. Pharmaceutical drugs analysed in this work by means of HS-GC-MS based on HTS-SUPRAS

Pharmaceutical formulation	Active pharmaceutical ingredient (API)	Excipients
Tablets	Paracetamol (1g)	Pregelatinized corn starch; Stearic acid; Povidone; Crospovidone; Microcrystalline cellulose and Magnesium stearate
Capsules	Magnesium metamizole (575mg)	Magnesium stearate. The components of the capsule are: Indigotine (E 132); Erythrosine (E 127); Titanium dioxide (E 171) and Gelatin
Suppositories	Magnesium metamizole (500mg)	Magnesium stearate; Gelatin; Red iron oxide; Erythrosine; Titanium dioxide
Powder	Paracetamol (500mg); Chlorphenamine Maleate (2mg); Phenylephrine Hydrochloride (7.5mg); Dextromethorphan Hydrobromide (10mg); Ascorbic Acid (200mg)	Sodium Saccharin; Sodium Cyclamate; Anhydrous Citric Acid; Mannitol (E-421); Sucrose; Disodium Edetate; Thiazolidine-carboxylic acid; Colloidal anhydrous silica; Povidone and Aroma-Cola Dye
Granules	Paracetamol (1g)	Aspartame (E-951); Sodium saccharin; Povidone; Anhydrous sodium carbonate; Sodium bicarbonate; Anhydrous citric acid; Anhydrous monosodium citrate and Lemon flavor
Suspensions	Amoxicillin trihydrate equivalent to 875 mg of amoxicillin and clavulanate potassium equivalent to 125 mg of clavulanic acid	Magnesium stearate; Sodium carboxymethylstarch (Type A) (from potato starch); Colloidal silica; Microcrystalline cellulose. Coating: titanium dioxide (E171); Hypromellose; macrogol (4000, 6000) and Dimethicone

Pharmaceutical formulation	Active pharmaceutical ingredient (API)	Excipients
Microspheres (capsule)	Calcium carbonate; Capsule (coating agent composed of gelatin and dyes E132 and E171); L-ascorbic acid; Magnesium oxide; DL-alpha tocopherol; filler (microcrystalline cellulose); Anti-caking agent (magnesium stearate); Nicotinamide; Sulphate iron; Calcium D-pantothenate; Zinc oxide; Retinyl acetate; Stabilizer (silicon dioxide); Manganese sulfate; Cholecalciferol; Pyridoxine hydrochloride; Phytomedione; Riboflavin; Thiamine hydrochloride; Copper sulfate; Cyanocobalamin; Teroylmonoglutamic acid; Picolinate of chromium; Sodium selenite; Potassium iodide; D-biotin.	
Aerosol	Miconazole (8.7mg)	Capro-caprylate glycerol and polyoxyethylene glycol; Polyethylene glycol 300 and alcohol 99.8%
Emulsion	Aqua; paraffinumliquidum, Glycol stearate; Propylene glycol; Stearic acid; Palmitic acid; Paraffin; Squalane; Perseagratissima oil; Triethanolamine; Algin; sodium sulfate; Cetyl palmitate; Cetyl stearate; Cetyl behenate; Cetyl laurate; Cetyl myristate; Sulfamic acid; Sodium polyphosphate; Potassium sorbate; Sodium methylparaben; Sodium propylparaben; parfum	
Injections (intravenous / intramuscular)	Benzylpenicillin sodium 600000 U.I. Benzathine benzylpenicillin 200000 U.I. Calcium phenoxymethylpenicillin 200000 U.I	Water

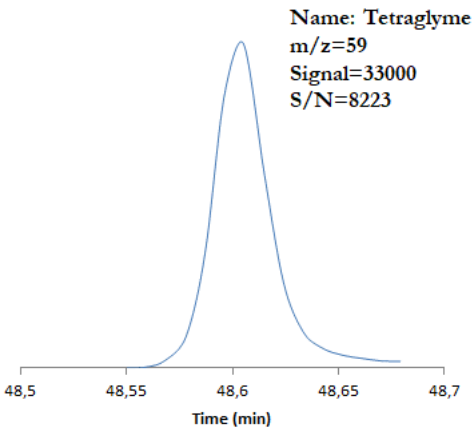
Table S3. Validation parameters for the Class 1, Class 2A and Class 2B residual solvents obtained by the proposed HS-GC-MS method based on HTS-SUPRAS

Class 1 solvent	Retention time / min	Linear range / %CL	DL / %CL	Repeatability (Intermed. precision) / %			Recoveries / %		
				50% CL	100%CL	150% CL	50% CL	100% CL	150% CL
1,1-Dichloroethene	4.1	30-250	10	10 (18)	9 (15)	7 (13)	108±15	90±7	104±11
Carbon tetrachloride	8.4	26-250	8.5	8 (14)	8 (14)	6 (13)	90±15	104±7	101±15
1,1,1-Trichloroethane	8.9	18-250	6.0	7 (13)	6 (16)	5 (15)	95±12	99±10	103±7
Benzene	10.0	26-250	8.5	8 (19)	9 (20)	6 (19)	111±14	95±8	96±8
1,2-Dichloroethane	10.1	26-250	8.5	10 (17)	10 (16)	5 (16)	104±12	93±7	105±10

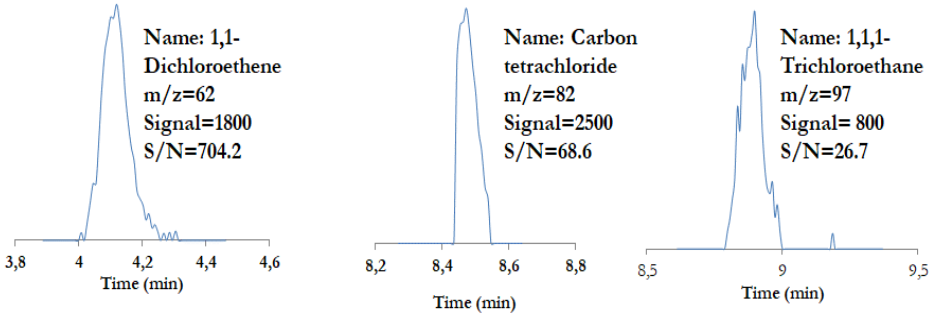
Class 2A solvent	Retention time / min	Linear range / %CL	DL / %CL	Repeatability (Intermed. precision) / %			Recoveries / %		
				50% CL	100%CL	150% CL	50% CL	100% CL	150% CL
Methanol	2.5	26-250	8.5	8 (12)	8 (15)	4 (10)	110±10	99±8	96±10
Acetonitrile	4.3	45-250	15	10 (11)	8 (16)	6 (12)	119±9	92±10	92±10
Dichloromethane	4.7	27-250	9.0	9 (10)	9 (11)	7 (9)	94±13	103±12	103±14
E-1,2-Dichloroethene	5.3	30-250	10	9 (15)	7 (15)	5 (11)	101±14	103±15	100±10
Z-1,2-Dichloroethene	7.5	24-250	8.0	10 (15)	10 (15)	7 (9)	99±11	105±9	97±10
Tetrahydrofuran	8.3	24-250	8.0	7 (16)	6 (12)	5 (9)	107±11	100±9	97±7
Cyclohexane	9.0	20-250	6.5	9 (13)	9 (11)	5 (10)	98±14	97±12	104±12

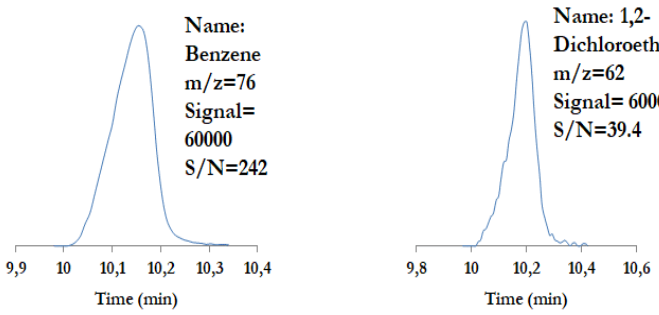
Class 2A solvent	Retention time / min	Linear range / %CL	DL / %CL	Repeatability (Intermed. precision) / %			Recoveries / %		
				50% CL	100%CL	150% CL	50% CL	100% CL	150% CL
Methylcyclohexane	13.0	28-250	9.5	9 (11)	9 (14)	8 (11)	109±8	100±15	95±14
1,4-Dioxane	13.9	20-250	6.5	10 (16)	9 (11)	4 (12)	98±13	102±13	97±7
Toluene	18.2	36-250	12	9 (15)	9 (12)	4 (14)	105±14	104±7	97±9
Chlorobenzene	27.0	7.5-250	2.5	9 (16)	8 (13)	5 (9)	102±13	100±7	99±7
Ethylbenzene	28.4	20-250	6.5	8 (10)	8 (14)	6 (9)	107±14	94±10	101±12
m-p-Xylene	29.6	21-250	7.0	9 (13)	5 (11)	4 (10)	105±10	96±8	102±13
o-Xylene	33.4	30-250	10	9 (12)	7 (13)	7 (9)	114±7	95±7	96±11
Cumene	35.9	16-250	5.5	10 (12)	7 (13)	6 (9)	111±10	96±7	95±8

Class 2B solvents	Retention time / min	Linear range / %CL	DL / %CL	Repeatability (Intermed. precision) / %			Recoveries / %		
				50% CL	100%CL	150% CL	50% CL	100%CL	150% CL
Hexane	5.9	21-250	7.0	6 (13)	6 (10)	5 (11)	106±7	96±10	98±15
Chloroform	8.5	39-250	13	10 (15)	6 (16)	5 (11)	104±13	97±8	104±8
Nitromethane	10.1	28-250	9.5	6 (13)	5 (13)	5 (14)	85±14	106±12	104±14
1,2-Dimethoxyethane	10.2	20-250	6.5	9 (15)	8 (15)	8 (11)	110±11	94±12	98±8
1,1,2-Trichloroethene	12.3	30-250	10	6 (16)	5 (10)	5 (14)	87±8	104±15	106±8
Pyridine	17.5	15-250	5.0	6 (11)	7 (16)	6 (10)	107±8	95±12	100±9
Methylisobutylketone	18.9	26-250	8.5	10 (12)	7 (11)	6 (9)	110±9	98±8	94±13
Methylbutylketone	22.5	16-250	5.5	8 (16)	7 (11)	6 (14)	98±7	97±12	104±13
Tetralin	43.3	18-250	6.0	10 (17)	8 (10)	6 (14)	109±8	97±15	97±10

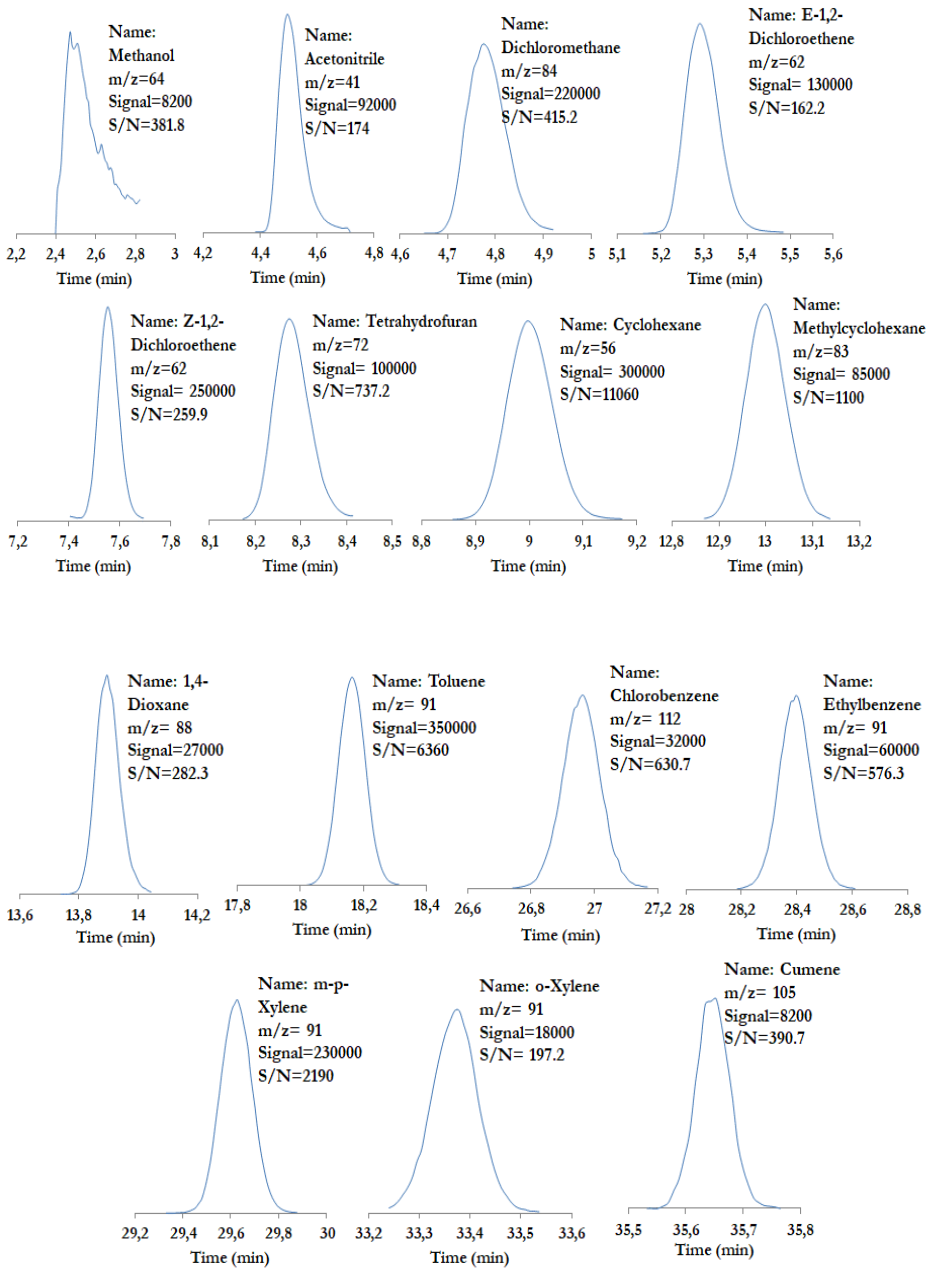


Internal standard





CLASS 1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S/N=381.8
Name: Acetonitrile
m/z=41
Signal=92000
S/N=174
Name: Dichloromethane
m/z=84
Signal=220000
S/N=415.2
Name: E-1,2-Dichloroethene
m/z=62
Signal= 130000
S/N=162.2
Name: Z-1,2-Dichloroethene
m/z=62
Signal= 250000
S/N=259.9
Name: Tetrahydrofuran
m/z=72
Signal= 100000
S/N=737.2
Name: Cyclohexane
m/z=56
Signal= 300000
S/N=11060
Name: Methylcyclohexane
m/z=83
Signal= 85000
S/N=1100
Name: 1,4-Dioxane
m/z= 88
Signal=27000
S/N=282.3
Name: Toluene
m/z= 91
Signal=350000
S/N=6360
Name: Chlorobenzene
m/z= 112
Signal=32000
S/N=630.7
Name: Ethylbenzene
m/z= 91
Signal=60000
S/N=576.3
Name: m-p-Xylene
m/z= 91
Signal=230000
S/N=2190
Name: o-Xylene
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Time (min)

CLASS 2A

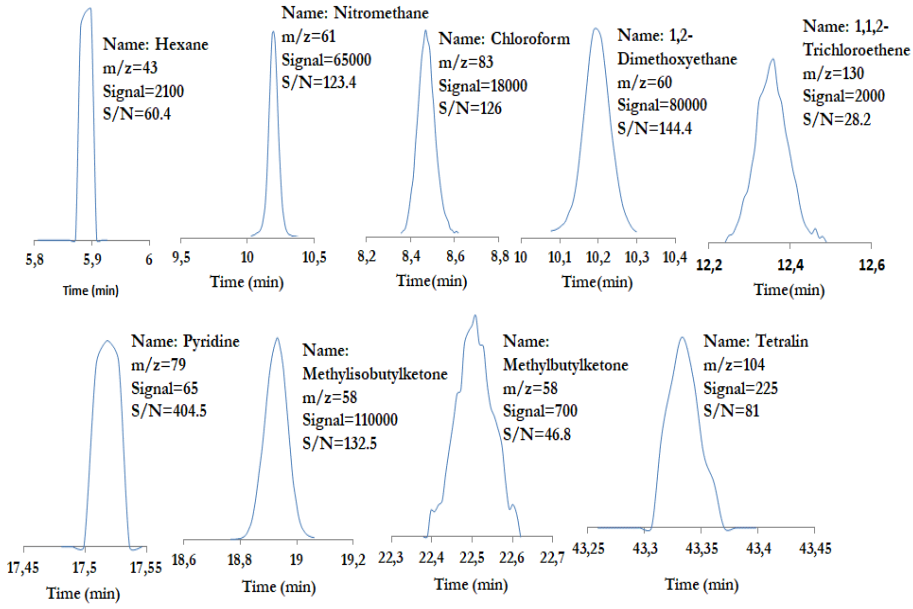

Figure S-1. Internal standard, class 1, class 2A and class 2B HS-GC-MS extracted ion chromatograms obtained from an HTS-SUPRAS spiked with the 37 residual solvents at their respective concentration limits

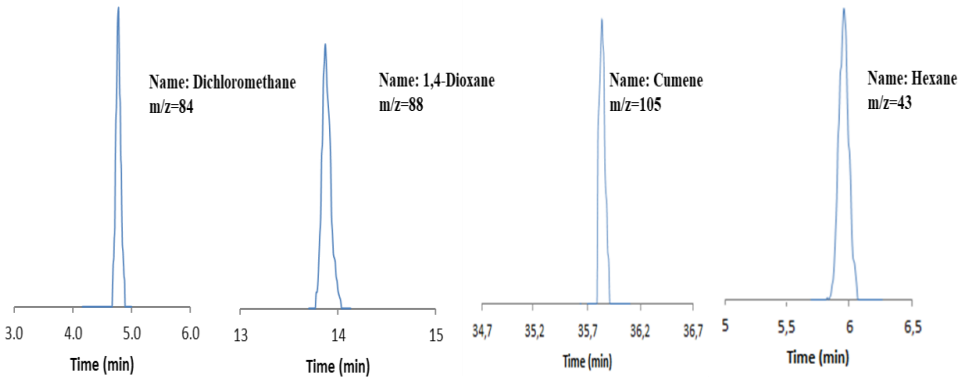

Figure S-2. HS-GC-MS chromatograms from an unspiked drug (formulation: suspension in Table S-2) analyzed by the method developed based on HTS-SUPRAS

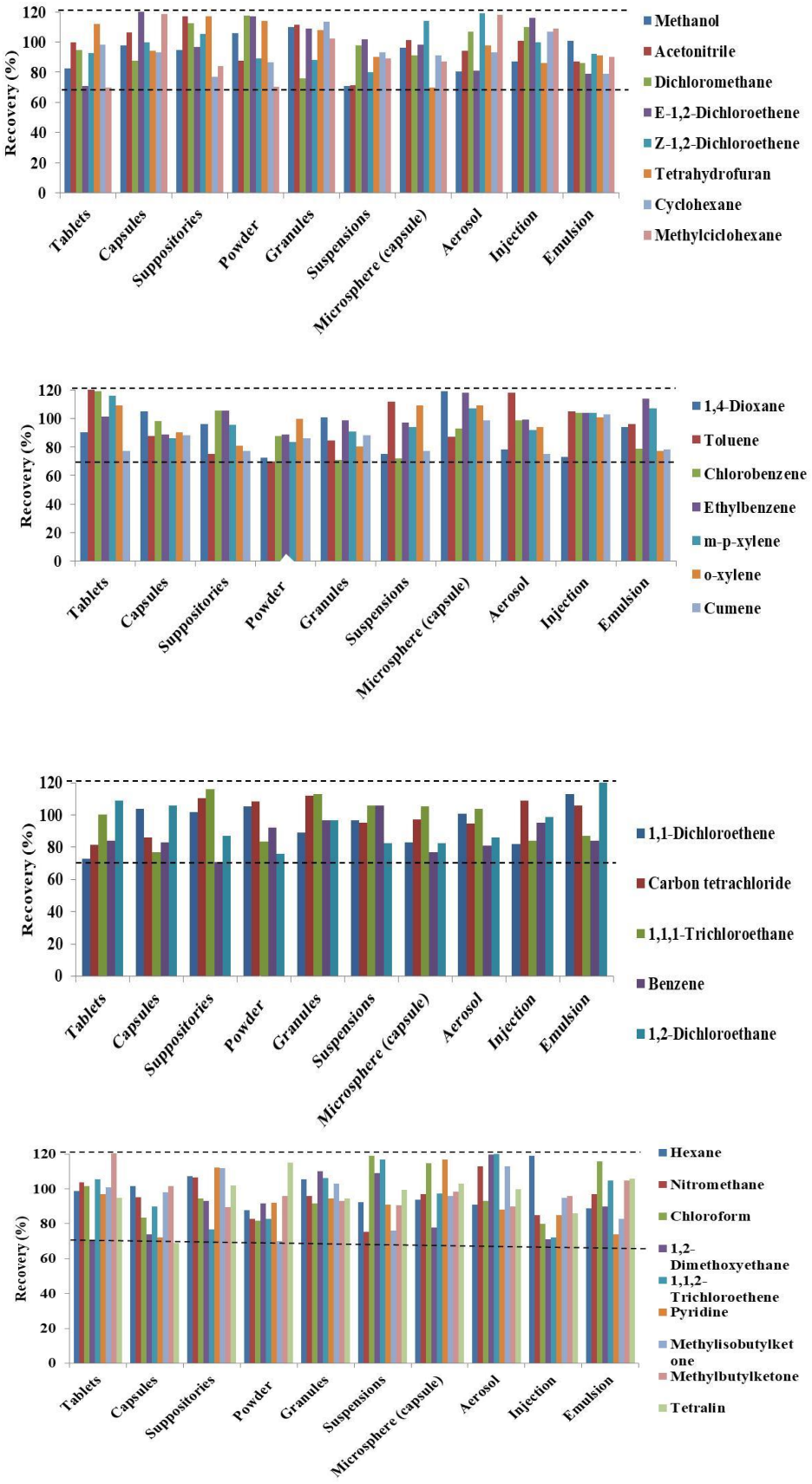

Figure S-3. Recoveries obtained for class 1, class 2A and class 2B residual solvents by the analysis of ten different drug formulations

Chapter IV

Multifunctional supramolecular solvents for combining extraction and encapsulation of lipophilic bioactive components

MULTIFUNCTIONAL GREEN SUPRAMOLECULAR SOLVENTS FOR COST-EFFECTIVE PRODUCTION OF HIGHLY STABLE ASTAXANTHIN-RICH FORMULATIONS FROM *Haematococcus pluvialis*

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Food Chemistry revision

HIGHLIGHTS

A novel combination of supramolecular solvents and nanostructured lipid carriers
Astaxanthin from *H. pluvialis* powder is extracted, encapsulated and stabilized
Spherical SUPRAS-NLCs of ~100 nm were obtained
Antioxidant and *in vitro* ROS scavenging properties are preserved and even enhanced
A remarkable time stability of at least 180 days at 4°C is guaranteed

KEYWORDS

Astaxanthin
Supramolecular solvents
Extraction
Encapsulation
Nanostructured lipid carriers
Haematococcus pluvialis

ABSTRACT

The interest of food industry to merchandise natural astaxanthin is growing up. However, it confronts scientific and technological challenges mainly related to its poor water solubility and chemical instability. Here, we present a new quick and efficient green process to simultaneously extract, encapsulate and stabilize astaxanthin from *Haematococcus pluvialis*. The process is based on the hither to unexplored combination of supramolecular solvents (SUPRAS), nanostructured liquids generated from amphiphiles

through sequential self-assembly and coacervation, and nanostructured lipid carriers (NLCs). These novel nanosystems were characterized by means of dynamic light scattering, AFM and cryo-SEM, revealing spherical particles of ~100nm. Their antioxidant activity was measured by ORAC (20.6±3.9 μM TE) and α-TEAC (2.92±0.58 μM α-TE) assays and their *in vitro* capacity to inhibit ROS by DHE probe. Results showed that the SUPRAS-NLCs proposed yield high extraction and encapsulation efficiencies (71±4%) in combination with a remarkable time stability (180 days, 4 ºC).

INTRODUCTION

In recent years, the use of dietary supplements and diet modifications towards a healthier model has increased. Among dietary supplements, there is a growing global demand for carotenoids (Landrum, 2010). Even though carotenoids obtained from chemical synthesis currently dominate the market (~76%), a strong growth rate (3.9%) is expected for natural carotenoids in response to the increasing consumer demand for natural products.

Production of natural carotenoids for application in the food industry confronts several scientific and technological challenges that mainly arise from their poor water solubility and chemical instability when exposed to light, heat and oxygen. Their instability in acidic environments is especially relevant upon digestion since they may be quickly destroyed in the stomach by the action of gastric fluids (Priyadarshani, 2017). The high lipophilicity of carotenoids implies the use of organic solvents for their extraction from vegetal biomass and the subsequent removal of the extraction solvent. Additionally, the chemical instability of carotenoids, resulting from its electron rich conjugated double bond structure, makes it difficult to keep their integrity during extraction, food processing and marketing.

Among carotenoids, astaxanthin is especially relevant because of its powerful antioxidant activity on the quenching of singlet oxygen (Focsan, Pan & Kispert, 2014) and peroxyl radicals (Müller, Fröhlich & Böhm, 2011). Natural astaxanthin is frequently obtained from the dried and crushed powder of the microalgae *Haematococcus pluvialis* by extraction with supercritical carbon dioxide (SFE) or with a mixture of polar and non-polar organic solvents (e.g. ethanol-hexane and chloroform-methanol (Saini & Keum, 2018)). SFE is advantageous, since it is a green technology, but the yield for astaxanthin

is low if organic modifiers, such as ethanol, are not added (Machmudah, Shotipruk, Goto, Sasaki, & Hirose, 2006). Furthermore, the SFE process is quite expensive, increasing the cost of production of natural astaxanthin and, therefore, favoring the use of cheaper sources. Extraction with organic solvents is relatively cheap and easy to scale up but it requires several extraction steps, high biomass-solvent ratios (usually 1:10, w/v), high extraction temperatures and long extraction times (24-48h) to give acceptable yields. Solvent-based extraction can be sped up with the use of pressurized liquids and may become greener using GRAS (generally recognized as safe) solvents (Jaime et al., 2010; Molino et al., 2018). However, solvent evaporation is mandatory, which increases process time and cost (Gong &Bassi, 2016) and deteriorates astaxanthin antioxidant activity (Zhao, Zhang, Fu, & Zhu, 2016). These drawbacks have fostered the research on greener and more efficient processes for the extraction of astaxanthin from *H. pluvialis*.

The limited stability of astaxanthin over time and/or during food processing is another major drawback for its application in the food industry. Stabilization of astaxanthin has been attempted by several strategies including spray-dried encapsulation (Janiszewska-Turak, 2017), incorporation into liposomes (Martínez-Delgado, Khandual, & Villanueva-Rodríguez, 2017), molecular inclusion (Yuan, Jin, &Xu, 2012), micro- and nano-encapsulation (Zhou et al., 2018) and O/W emulsions (Zhang et al., 2017). Recently, nanostructured lipid carriers (NLCs) have been proposed as promising candidates for delivering bioactive molecules in food applications (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2013). They are lipid-in-water emulsions containing a mixture of a solid lipid (e.g. stearic acid, glyceryl monostearate, cetyl palmitate, glyceryl behenate, etc.), a liquid lipid (e.g. medium chain triglycerides, soybean oil, oleic acid, etc.) and emulsifiers (e.g. poloxamer 188, tween 80, lecithin, etc.) (Tamjidi et al., 2013). It has been shown that degradation of astaxanthin, in the best formulation obtained for NLCs, is around 20% after 25 days of storage. Further research on astaxanthin-loaded NLCs has focused on the development of strategies to increase their stability (Tamjidi, Shahedi, Varshosaz & Nasirpour, 2014), the influence of food components and processing parameters on their integrity (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2017), and their behavior in beverage systems (Tamjidi, Shahedi, Varshosaz, &Nasirpour, 2018).

Because of the low stability shown by astaxanthin, its transfer from vegetal biomass to food should be ideally carried out under mild conditions through a quick

and efficient green process. In this way, in this manuscript we propose a supramolecular solvent (SUPRAS) as an astaxanthin transfer liquid phase vector with multifunction tasks, namely, extraction, encapsulation and stabilization. For this purpose, a SUPRAS will be designed to extract astaxanthin from *Haematococcus pluvialis* and produce oleoresins that will be directly used as the liquid lipid ingredient to produce astaxanthin-loaded nanostructured lipid carriers (NLCs). Based on our extensive experience on SUPRASs (Ballesteros-Gómez, Sicilia, & Rubio, 2010), we hypothesize that they can be tailored to maximize astaxanthin stability while maintaining process yield and selectivity.

SUPRASs are nanostructured liquids made up of three-dimensional aggregates of amphiphiles (e.g. carboxylic acids, alkanols, alkyl sulfates, alkyl phenols, etc.) that are synthesized from colloidal solutions by self-assembly and coacervation (Caballo, Sicilia, & Rubio, 2017). The amphiphiles arrange in the SUPRAS as three-dimensional aggregates via non-covalent bonds giving a variety of possible morphologies that are environment responsive (Ballesteros-Gómez & Rubio, 2012). SUPRASs have intrinsic properties that makes them excellent candidates for the development of efficient extractions. They offer microenvironments of different polarity, high concentration of binding sites and high surface area due to their discontinuous character. Accordingly, fast and efficient extractions can be achieved using low SUPRAS volumes. An outstanding property of SUPRASs is that they can be tailored to give specific functions, thus enabling the possibility of increasing efficiency and selectivity (Ballesteros-Gómez & Rubio, 2012). The ability of SUPRASs to efficiently extract carotenoids from vegetal biomass has already been proved by means of a proprietary extraction process that gives carotenoid-rich SUPRAS oleoresins with similar composition to those obtained from SFE, but at a considerable lower cost (Rubio et al., 2017). Summing up, here we present and explore the hypothesis of supramolecular aggregates (SUPRASs) being an excellent and novel vector to simultaneously extract, encapsulate and stabilize astaxanthin from *H. pluvialis* for its subsequent application in food industry as NLCs. This approach should be based on the careful selection of a SUPRAS amphiphile that should maximize several different interactions with astaxanthin while showing a structure that may enhance stabilization by avoiding oxidation.

MATERIAL AND METHODS

Materials

All chemicals were of analytical-reagent grade and employed as supplied. The following reagents were purchased from Sigma-Aldrich (Steinheim, Germany): octanoic acid, stearic acid, poloxamer 407, poloxamer 188, potassium persulfate, 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (TROLOX), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), α-Tocopherol, N-acetyl-L-cysteine (NAC; A9165), antimycin A (*Streptomyces sp.*), dihydroethidium (DHE), fluorescein and astaxanthin (synthetic). Glyceryl palmitostearate (Precirol® ATO 5) was kindly offered by Gattefossé (Nanterre, France). Ethanol absolute (EtOH), acetone, dichloromethane and hydrochloric acid were from Carlo Erba (Val-de-Reuil, France). Ynsadiet Laboratories (Leganés, Spain) supplied soya lecithin. Ultra-pure water was produced in a Millipore (Billerica, MA, USA) purification system. Sodium hydroxide (NaOH) and potassium chloride (KCl) were from Merck (Darmstadt, Germany). All cell culture reagents and Dulbecco´s phosphate buffered saline (PBS) were all purchased from Gibco Technologies (Paisley, United Kingdom). 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was fromInterchim (Montluçon, France). Astaxanthin-containing (maximum 3% (w/w)) *Haematococcus pluvialis* powder (HPAx), a microalgae strain from the coast of Chile, was provided by Pigmentos Naturales (Pica, Chile) and was stored at -20ºC to avoid any astaxanthin degradation.

Synthesis of an octanoic acid-based supramolecular solvent (SUPRAS)

A volume of SUPRAS of around 13 mL was synthesized by dissolving 4mL of octanoic acid in 28mL of EtOH (100 mL centrifuge tubes) and adding 48mL of a 10mM HCl solution to induce coacervation. The mixture thus obtained was vortex-shaken at 2300 rpm for 7 minutes and then centrifuged to enhance phase separation (3500 rpm, 10 minutes, 25ºC). Two phases were obtained: the supramolecular solvent (at the top) and the equilibrium solution. Both phases were separated by collecting the upper phase with a Pasteur pipette and stored at 4 ºC in hermetically closed tubes until further use. The volume of SUPRAS obtained can be tuned at will by increasing the amount of octanoic acid used for the synthesis while keeping a constant water/EtOH

ratio (Rubio et al., 2017). This SUPRAS was subsequently used for the extraction of astaxanthin for *H. pluvialis* ($SUPRAS_{(oleoresin)}$, see section) or used directly for NLCs synthesis ($SUPRAS_{(blank)}$).

SUPRAS extraction of astaxanthin from *Haematococcus pluvialis*

HPAx was wetted with the equilibrium solution obtained in section and, immediately, the SUPRAS was added. The ratio HPAx:equilibrium solution:SUPRAS was 1:5:2 (g:mL:mL). The mixture was vortex-shaken for 8 minutes at 2300 rpm. Afterwards, it was centrifuged at 10000 rpm for 10 minutes obtaining three different phases: the biomass residue at the bottom, the equilibrium solution in the middle and the SUPRAS at the top. The SUPRAS phase was an oleoresin ($SUPRAS_{(oleoresin)}$) containing the carotenoids and the lipid fraction of the HPAx that was directly used for the synthesis of NLCs. The whole procedure was carried out in the dark. $SUPRAS_{(oleoresin)}$ was analyzed by means of HPLC-UV using a Ultrabase C18 (5 μm, 250 x 4.6 mm) column thermostatted at 20 ºC. Mobile phase consisted of an acetone:water mixture from 83:17 to 98:2 (%, v:v) in 80 minutes. Diode-array detector was set in the range 200-800 nm.

Synthesis of NLCs from SUPRAS

The synthesis of SUPRAS-NLCs was based on the "Hot Homogenization method" (Souto, Wissing, Barbosa, & Müller, 2004). The general procedure was as follows: an aqueous phase was prepared by dissolving 450 mg of a hydrophilic non-ionic surfactant (Poloxamer 188 or 407) in 15 mL of ultrapure water at 65°C. In parallel, an oil phase composed of 450 mg of a lipid (Precirol ATO5 or Stearic acid), 60 mg of a surfactant (Soya lecithin) and 750 μL of $SUPRAS_{(oleoresin)}$ or $SUPRAS_{(blank)}$ was heated at 65°C and homogenized by mechanic stirring at 10000 rpm for 2 minutes (PT-3100D Polytron). Then, the aqueous phase was added to the oil one drop wise for 10 minutes while stirring the mixture at 20000 rpm, adjusting afterwards the pH of the mixture to 8.0 with NaOH 2M. This mixture was finally stored in the dark at 4 ºC. After 24 hours, the mixture was centrifuged for 13 minutes at 13000 rpm until two phases were obtained: a suspension of SUPRAS-NLCs, and a precipitate consisting of unreacted ingredients, which was discarded.

SUPRAS-NLCs characterization

SUPRAS-NLCs, both containing astaxanthin from HPAx ($SUPRAS_{(oleoresin)}$-NLCs) and blanks ($SUPRAS_{(blank)}$-NLCs), were characterized in terms of encapsulation efficiency (EE), encapsulation loads (EL), zeta-potential, particle size, polydispersity index (PDI) and morphology.

Encapsulation load and efficiency

Encapsulation efficiency (EE) and loads (EL) were calculated based on the equations described by Fathi and Varshosaz (Fathi & Varshosaz, 2013):

$$EE(\%) = \frac{Astax_i}{Astax_t} * 100$$

$$EL(\%) = \frac{Astax_i}{Lipid} * 100$$

where "$Astax_i$" is the amount of astaxanthin incorporated into the $SUPRAS_{(oleoresin)}$-NLC,"$Astax_t$" the total amount of astaxanthin added as $SUPRAS_{(oleoresin)}$ and "Lipid" the total amount of lipids used for the preparation of NLCs, including liquid (octanoic acid) and solid (stearic acid or Precirol ATO 5) lipids. In order to determine $Astax_i$, the $SUPRAS_{(oleoresin)}$-NLCs obtained in subsequent section were dissolved (dilution factor 1/10) in a mixture of solvents (ethanol:dichloromethane:water) in a volume ratio of 90.5: 6.0: 3.5. Later, these solutions were diluted in acetone (dilution factor 1/10) and their absorbance measured at 480 nm in a spectrophotometer (Safas Monaco, France). To determine $Astax_t$, the $SUPRAS_{(oleoresin)}$ obtained in subsequent section was diluted in acetone (dilution factor 1/1000) and the absorbance measured at 480 nm. Quantification of both $Astax_i$ and $Astax_t$ was carried out by determining their concentrations by means of a calibration curve constructed from solutions of pure synthetic astaxanthin in acetone within the concentration range of 10-50μM (ε=0.092$cm^{-1}\mu M^{-1}$, R^2 = 0.9999). Measurements were carried out in triplicate.

Particle size, polydispersity and zeta potential

SUPRAS-NLCs were diluted 1/100 in water for particle size and polydispersity index determination and 1/10 inKCl 1mM for zeta potential measurements. Before analysis, all samples were sonicated for 10 minutes. Size (nm), polydispersity index (PDI), and average zeta potential (mV) were determined using dynamic light scattering (Zetasizer Nano ZS, Malvern instrument, Worcestershire, United Kingdom). Temperature was controlled at 25°C and the analysis was performed in triplicate for each sample.

Morphology of SUPRAS-NLCs

The morphology of the SUPRAS-NLCs was studied by AFM and Cryo-SEM microscopy. For AFM microscopy, aliquots of SUPRAS-NLCs were diluted in water (1/40) and coated by means of a spin coating procedure (1.500rpm, 30 s, $500s^2$) with a spin coating Spin 150i (Midden Engweg 41NL -3882 TS Putten, Netherlands). Once the samples were ready, they were immediately introduced in the AFM microscope (Bruker France SAS, 91120 Palaiseau, France) to determine their morphology and distribution. For Cryo-SEM microscopy (JOEL, JSM-6700F, Japan), SUPRAS-NLCs were processed as described by Anne Saupe et al. (Saupe, Gordon, & Rades, 2006). Briefly, a drop of dispersed sample was filled into a brass rivet and plunge frozen in liquid nitrogen slush. Samples were then transferred into the cryo stage (Gatan, Alto 2.500, UK) where they were fractured. All samples were investigated 90 days after preparation.

Study of the antioxidant capacity of SUPRAS-NLCs

In order to study the antioxidant capacity of SUPRAS-NLCs, all the antioxidants investigated (controls, $SUPRAS_{(olerorresin)}$, $SUPRAS_{(oleoresin)}$-NLCsand $SUPRAS_{(blank)}$-NLCs) were prepared at the concentrations specified in other sections in a mixture of EtOH/DCM/H_2O (90.5/6.0/3.5, % v/v). Two different tests were run to measure their oxygen radical absorbance capacity (ORAC) and α-Tocopherol Equivalent Antioxidant Capacity (α-TEAC).

Oxygen Radical Absorbance Capacity (ORAC)

Antioxidant activity ofSUPRAS-NLCswas evaluated bythe following procedure (Cao, Alesio, & Cutler, 1993): 25µL of control ($SUPRAS_{(oleoresin)}$ 0-20 µM), standard (Trolox 0-100 µM), $SUPRAS_{(oleoresin)}$-NLCs(0-20 µM) and $SUPRAS_{(blank)}$-NLCs(0-20 µM) were added to a fluorescein solution (150µL, 4 nM in PBS). Then, the radical reaction was initiated by addition of 25 µL of AAPH (153 mM in PBS) and the fluorescence decay at 37°C (λex/λem485-528 nm) was monitored during 1 h (TECAN Männedorf, Switzerland). Final ORAC values were calculated by using the regression equation between the linear portion of the curve obtained for Trolox concentration 0-50 µM and the net area under the curve (AUC) and were expressed as µM Trolox equivalents (µM TE). As an example, ORAC for $SUPRAS_{(oleoresin)}$-NLCs was calculated as stated by equations 1 and 2:

$$AUC_{net} = AUC_{SUPRAS(oleresin)\text{-}NLCs} - AUC_{SUPRAS(blank)\text{-}NLCs} \quad (1)$$

$$ORAC(\mu MTE) = \frac{(SUPRAS_{(oleresin)}\text{-}NLCs) - (SUPRAS_{(blank)}\text{-}NLCs)}{TROLOX} \quad (2)$$

where $SUPRAS_{(oleoresin)}$-NLCs, $SUPRAS_{(blank)}$-NLCs and Trolox are the slopes obtained for the net AUC for each sample versus the concentration in µM.

α-Tocopherol Equivalent Antioxidant Capacity (α-TEAC)

The lipophilic α-TEAC method (Apak et al., 2013) used in this work was based on ABTS assay that measures the capacity of a sample to scavenge the ABTS radical ($ABTS^{+}$). $ABTS^{+}$ stock solution (7mM) was generated by oxidation of ABTS (14mM in distilled water) by potassium persulfate ($K_2S_2O_8$, 4.9mM in water) by mixing both solutions in a volume ratio of 1:1. A working solution ($ABTS^{+}$) was prepared by dilution of the stock solution in PBS (pH 7.4) until an absorbance of 0.7-0.8 at 734nm was reached (Spectrophotometer UV mc1, SAFAS MC 98000 Monaco). Solutions of α-Tocopherol (lipophilic standard reference), $SUPRAS_{(oleoresin)}$, $SUPRAS_{(oleoresin)}$-NLCsand $SUPRAS_{(blank)}$-NLCs were all prepared in EtOH/DCM/H_2O (90.5/6.0/3.5, % v/v) at concentrations in the range 0-50 µM. 300µL of each antioxidant were added to 1mL of $ABTS^{+}$ and stirred for 45 minutes to be finally centrifuged (2 minutes 10000rpm). α-

TEAC values were calculated as α-Tocopherol equivalents (μM Astaxanthin / μM α-Tocopherol). Each sample was analyzed in triplicate.

Time stability studies

Stability studies of the SUPRAS-NLCs were carried out for a period of 180 days. These studies were based on the methodical determination of the encapsulation efficiency (EE, %), their anti-oxidant capacity (α-TEAC), size and zeta potential. These last two parameters were stated as described in subsequent section. During this period SUPRAS-NLCs were conserved at 4ºC in the dark.

Evaluation of endothelial cell protection by SUPRAS-NLCs

Cell culture

Human umbilical vein endothelial cells (HUVEC, ATCC CRL 1730) were cultured in minimum essential Medium-L-Glutamine (MEM), supplemented with 10% (v/v) fetal calf serum (FBS) and 1% penicillin–streptomycin-amphotericin (PSA). Cells were seeded in a T75 cell culture flask and kept in a humidified incubator containing 5% CO_2 at 37°C. The culture medium was replaced twice every week and the cells were split 1:3 every week.

Determination of reactive oxygen species (ROS) using DHE probe.

Prior to tests, HUVEC were removed from growth media and detached with trypsin after 80% of confluence. A density of 10^4 cells/well were seeded in 96-well cell culture plates and incubated overnight at 37°C with 5% CO_2. 25μL of $SUPRAS_{(oleoresin)}$-NLCs(1.6μM) and $SUPRAS_{(blank)}$-NLCs (4.6mg/mL) were added to cells and incubated during 24, 48 or 72 h. Then, cells were washed twice with PBS to remove the medium and incubated in the dark with DHE (5 μM), for 30 minutes at 37°C. N-acetyl cysteine (NAC, 300 mM) reference antioxidant was added to the negative controls. Cells were gently washed three times with warm buffer. Reactive oxygen species were induced by the addition of the stressor Antimycin A (AA; 200 μM, during 30 minutes), excluding the blank wells. Fluoresce was monitored at λex/λem = 500/580 nm (TECAN

Männedorf, Switzerland). Antioxidant activities were expressed as ROS scavenging efficiency:$RSE\ (\%) = (1 - F_{sample}/F_{Antimycin}) \times 100$.

Statistical analysis

All experiments were repeated at least three times to ensure the reproducibility of each test. Results were expressed as the mean ± SDE and statistical analyses were done using one-way ANOVA followed by Tukey's HSD post hoc test (JMP Software, Version 9; SAS Institute, Cary, NC, USA). Results were considered to be significant if p-value < 0.05.

RESULTS AND DISCUSSION

SUPRAS selection for HPAx extraction, encapsulation and stabilization

In order to fit the objectives of this research, the SUPRAS selected for integrating both extraction and encapsulation of HPAx should meet, at least, the following requirements: high extraction yields, compatibility of the SUPRAS extracts with NLC formation, and astaxanthin stabilization. For this purpose, a good knowledge of both, sample composition and chemical structure of constituents, is mandatory.

HPAx (EFSA, 2014) typically consists of fat (45-50%), protein (9-12%), carbohydrates (20-30%), dietary fibre (10%) and ash (1.5-2.5%). The content of total carotenoids varies in the range from 5.2 to 5.8% and it is mainly made up of astaxanthin (~95%) in three forms (~2% free, ~80% monoesters and ~18% diesters, see structures in Figure 1.A). The remaining 5% of carotenoids consists of canthaxanthin, β-carotene, lutein, zeaxanthin and others. The fatty acid fraction mainly contains linoleic (C18:2 cis, ~31%), oleic (C18:1 cis, ~24%), γ-linolenic (C18:3 cis, ~15%), palmitic (C16:0, ~12%) and eicosatrienoic (C20:3 cis, ~8%) acids.

In order to get high extraction yields, astaxanthin should stablish strong chemical interactions with the SUPRAS. Furthermore, SUPRAS extracts should mainly contain lipids and carotenoids to be compatible with NLCs. Accordingly, any other components from HPAx should be excluded from extraction.

With all these requirements in mind, a SUPRAS made up of octanoic acid, ethanol and water was investigated for extraction of astaxanthin (Rubio et al., 2017). Among natural amphiphiles, octanoic acid was selected for SUPRAS formation because it mimics astaxanthin structure, i.e. the length of two octanoic acid molecules closely resemble that of a molecule of astaxanthin (Figure 1.A), it is a saturated lipid, which may result in an enhanced stability against lipid peroxidation (Yin, Xu & Porter, 2011), and fatty acids have been approved as food additives (Commission Regulation EU, 2011). Octanoic acid has been previously reported to form supramolecular solvents in a variety of binary mixtures of organic solvent-water, including ethanol, acetone, methanol, acetonitrile, tetrahydrofuran, etc. (Ruiz, Rubio & Pérez-Bendito, 2007). Ethanol was here selected as the organic solvent because it is considered as a GRAS solvent compatible with food and nutraceutical processing.

The formation of the octanoic acid-based SUPRAS is quite simple; octanoic acid solubilizes in ethanol forming reverse aggregates above the critical aggregation concentration. Then, water is added to the organic colloidal solution as a coacervation-inducing agent. The addition of water causes the increase of the supramolecular aggregates, which form oily droplets that associate in clusters of individual droplets. The density of these conglomerates is different from that of the solution in which they formed, that facilitating their creaming and separation as a new liquid phase, named SUPRAS (Ballesteros-Gómez et al., 2010; Rubio et al., 2017). The SUPRAS is in equilibrium with an ethanol:water solution containing octanoic acid below the critical aggregation concentration. Figure 1.B shows the phase diagram obtained for ternary mixtures of ethanol, water and octanoic acid and depicts the boundary for the SUPRAS formation region (Rubio et al., 2017).The molecules of octanoic acid in the SUPRAS arrange as inverted hexagonal aggregates where the polar groups surround aqueous cavities and the hydrocarbon chains are dispersed in ethanol (Figure 1.B) (Ballesteros-Gómez & Rubio, 2012; Caballo et al., 2017). Because only the protonated form of octanoic acid undergoes coacervation (pKa = 4.75), the formation of this SUPRAS is carried out at pH < 3.

Carboxylic acid-based SUPRAS are environment responsive (Caballero-Casero et. al., 2015; Ballesteros-Gómez et al., 2012) and both, SUPRAS composition and size of the aqueous cavities of the inverted hexagonal aggregates, can be tailored by modifying the proportion of ethanol and water in which octanoic acid self-assembles. Thus, the water content in the SUPRAS increases, and consequently the size of their water cavities

also does, as the ethanol/water ratio rises (Figure 1.B). This means that this SUPRAS has the potential to be have as a restricted access liquid for polar macromolecules such as polysaccharides that are size-excluded from the vacuoles. Moreover, proteins precipitate because of the decrease of the dielectric constant of the solution caused by ethanol and the formation of macromolecular complexes with octanoic acid (Caballero-Casero et al., 2015). All these properties result in a SUPRAS with a high potential to produce extracts compatible with NLCs.

Figure 1.A shows a schematic of the SUPRAS extraction of astaxanthin from HPAx and the chemical and physical mechanisms involved in astaxanthin solubilization and oleoresin production. The inverted hexagonal aggregates making up the SUPRAS offer a twofold mechanism for solubilization of astaxanthin, namely dispersion interactions in the hydrophobic tails and hydrogen bonds in the polar head groups and aqueous cavities. Astaxanthin contains hydrogen donors and acceptors, and a hydrocarbon chain whose length matches that of two octanoic acid molecules, so astaxanthin perfectly fit in the SUPRAS structure. Therefore, the forces driving extraction are expected to be both dispersion interactions and hydrogen bonding (Figure 1A).

Extraction of astaxanthin with SUPRAS from HPAx was investigated as stated in the experimental section. A ratio biomass:equilibrium solution: SUPRAS of 1:5:2 was considered optimal for proper biomass humidification and quantitative recovery of astaxanthin. A high extraction yield (96 ± 7 %) was obtained, including all forms of astaxanthin (free, monoesters and diesters), which can be attributed to the mixed mode mechanism able for their solubilization. As a comparison, extraction yields for SFE, using ethanol or vegetable oil as modifiers, are in the intervals 51-97% (Reyes et al. 2014). The maximum astaxanthin content expected for $SUPRAS_{(oleoresin)}$in this research was around 1.5% (w/v) taking into account the maximum content of astaxanthin in the HPAx investigated (around 3%), the SUPRAS/sample ratio used(2) and the recovery obtained (~100%). It should be noted that proteins gave a white precipitate that separated from the equilibrium solution as a thin layer upon centrifugation (Figure 1A). The analysis of the SUPRAS extract by liquid chromatography coupled to UV detection gave a profile for carotenoids that, as expected, was similar to that obtained for supercritical fluid extraction (Figure 1.C). The $SUPRAS_{(oleoresin)}$ obtained in this way was subsequently used for the synthesis of NLCs.

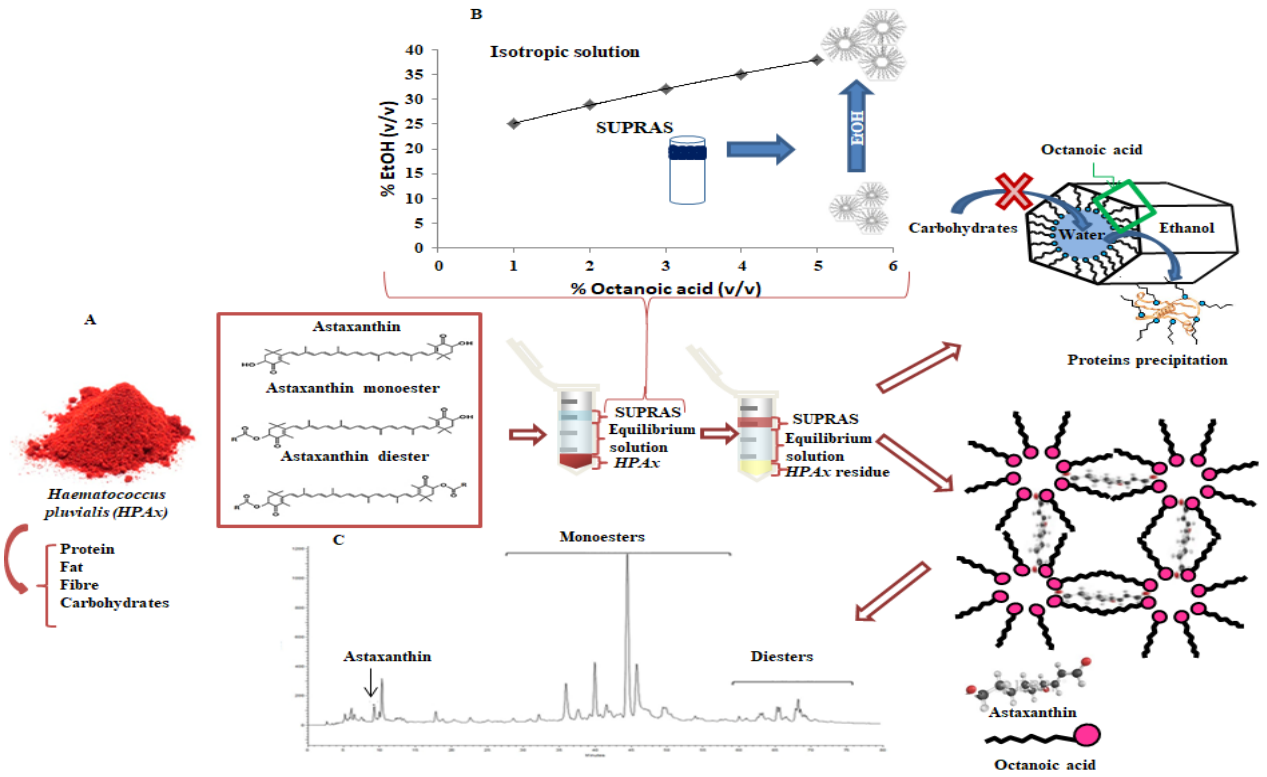

Figure 1. Scheme of SUPRAS synthesis, extraction and stabilization of astaxanthin from HPAx (A), SUPRAS phase diagram and structure (B), and astaxanthin-containing SUPRAS extract LC-MS profile (C)

Optimization of SUPRAS-NLCs synthesis

In order to find the best formulation for encapsulation of astaxanthin, we studied four different formulations, while keeping the same synthesis protocol starting from $SUPRAS_{(oleoresin)}$: two aqueous surfactants (i.e., Poloxamer 188 and 407) and two solid lipids (i.e., Precirol ATO5 and stearic acid) were tested. In all cases, the lipid phase surfactant was lecithin soya. These four formulations (S-NLC1 to S-NLC4) are presented in Table 1.

Table 1 also presents the DLS characterization (Size, PDI and Zeta potential) and the results obtained in terms of encapsulation load and encapsulation efficiency for all formulations, analyzed by means of a two-factor ANOVA. Depending on the composition, the particle sizes observed varies in a range from 97 to 426 nm (Table 1). In terms of solid lipid, stearic acid leads tothe formation ofsignificantly smaller and less dispersed (P-values of 0.028 and 0.023, respectively) nanoparticles than Precirol ATO 5. With this first data, the syntheses S-NLC1 and S-NLC 3 were discarded because NLCs with asmaller size and PDI are preferred based on the fact that 100nm is the most suitable size for oral drug delivery, exhibiting highest C_{max} and $AUC_{0\text{-}24h}$ than NLCs of larger diameters (Li, Chen, Su, Sun & Ping, 2016). Moreover, even though S-NLC 2 showed a significantly better zeta potential than S-NLC 4, this one was slightly superior in terms of encapsulation load (24% vs. 19%) and efficiency (71% vs 58%). Thus, it was finally decided to choose S-NLC 4, which presents a more favorable size and it is close to optimal values for the rest of variables studied. Based on these results, this formulation (from now on SUPRAS-NLCs) was used for all following studies.

Morphology of SUPRAS-NLCs

The morphology of the SUPRAS-NLCs obtained by the optimized formulation was studied by two microscopy techniques, AFM and cryo-SEM, as stated in the experimental section. It was proved that SUPRAS-NLCs shows a spherical type, with sizes that coincide with those obtained by DLS, giving accuracy and coherence to the results obtained throughout that characterization stage (see section above). Figure 2 shows the morphology of the nanoparticles in terms of DLS measurements (2.A and B) and AFM microscopy (2.C and D) for $SUPRAS_{(oleoresin)}$-NLCs and $SUPRAS_{(blank)}$-NLCs.It should be highlighted that the SUPRAS-NLCs synthetized from $SUPRAS_{(oleoresin)}$ maintain the spherical shape observed in AFM microscopy for those synthetized from

$SUPRAS_{(blanks)}$, as well as polydispersity and stability. On the other hand, $SUPRAS_{(oleoresin)}$-NLCs (112 nm) are larger than $SUPRAS_{(blank)}$-NLCs (64 nm), which may be the result of astaxanthin being incorporated into SUPRAS structured (Figure 1). Cryo-SEM microphotographs resulted in no significantly different results (data not shown).

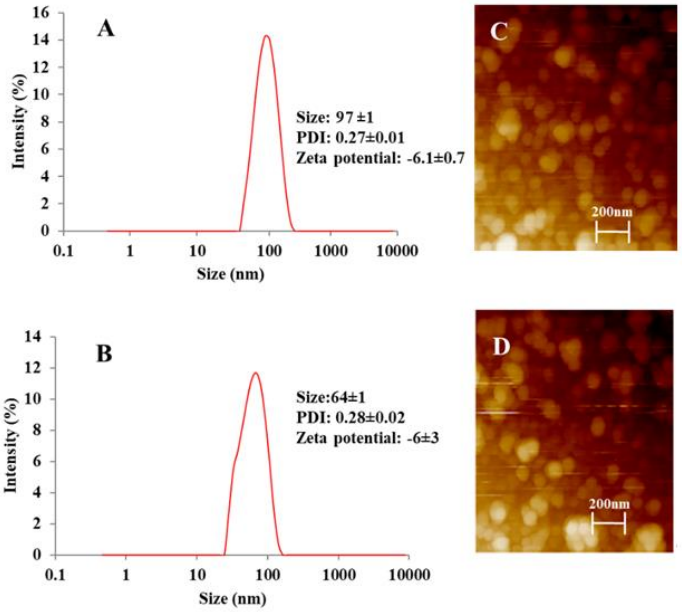

Figure 2. Size distribution charts obtained by DLS for $SUPRAS_{(oleoresin)}$-NLCs (A) and $SUPRAS_{(blank)}$-NLCs (B), and their respective AFM microscopy photographs (C-D)

Antioxidant capacity of SUPRAS-NLCs

The antioxidant activity of astaxanthin encapsulated in $SUPRAS_{(oleoresin)}$-NLCs was directly assessed by α–TEAC and ORAC tests. The first one is considered as a mixed hydrogen atom transfer (HAT)/electron transfer (ET)-based assay where as ORAC is only a HAT-based assay (Apak et al. 2013). Figure 3.A-C show the results obtained. Only the linear portion of the inhibition curves for ORAC (Fig. 3.B) and α-TEAC (Fig. 3C) were plotted.

Table 1. Composition and properties of the four different SUPRAS-NLCs studied in this work

Sample		Aqueous phase surfactant	Solid lipid	Lipid phase surfactant	Size (nm)	PDI	Zeta potential (mV)	Encapsulation load (%)	Encapsulation efficiency (%)
S-NLC 1		Poloxamer 188	Precirol ATO 5	Lecithin soya	295±43	0.42±0.08	-29.4±0.2	30±4	106±15
S-NLC 2		Poloxamer 188	Stearic acid	Lecithin soya	181±5	0.31±0.03	-28±2	19±2	58±6
S-NLC 3		Poloxamer 407	Precirol ATO 5	Lecithin soya	426±14	0.49±0.07	-8.6±0.4	20±4	59±13
S-NLC 4		Poloxamer 407	Stearic acid	Lecithin soya	97±1	0.27±0.01	-6.1±0.7	24±1	71±4
P-value[a]	**APS**				0.993	0.988	0.016*	0.795	0.672
	SL				0.028*	0.023*	0.175	0.722	0.656

a) P-value for a two-factor analysis of variance (ANOVA, Statgraphics Centurion 16.1.03). APS: aqueous phase surfactant; SL: solid lipid.

ORAC method is based on the inhibition of alkyl (R·), peroxyl (ROO·), andalkoxyl (RO·) radicals, where R=$H_2N(HN)C$ (Caoet al., 1993) produced by thermal decomposition of 2,2'-azobis(2-amidino-propane) (AAPH). In this test, the fluorescence intensity of fluorescein decreases due to the action of AAPH. The protective effect of astaxanthin was measured by assessing the kinetics of fluorescence decay *vs* time (Figure 3.A) compared to a standard antioxidant (Trolox) and expressed as μM Trolox equivalent (TE). By analyzing the AUC Net, the $SUPRAS_{(oleoresin)}$calibration curve presented a slope of 6.66 whilst it was 0.44 for Trolox, thus representing a scavenging activity of 15.1±2.7 μM TE. After inclusion in $SUPRAS_{(oleoresin)}$-NLCs, an increase in the antioxidant activity was observed (Figure 3.B). In order to take into account only astaxanthin activity, AUC Net values obtained for $SUPRAS_{(blank)}$-NLCs were subtracted to those of the $SUPRAS_{(oleoresin)}$-NLCs (Figure 3.A and 3.B). The obtained ORAC activity for $SUPRAS_{(oleoresin)}$-NLCs was 20.6±3.9 μM TE.

The antioxidant activities of samples were also measured asα-TEAC (ABTS assay) and expressed as μM α-Tocopherol equivalent (α-TE). The results (Figure 3.C) shown an increase of scavenging capacities of $SUPRAS_{(oleoresin)}$after inclusion in $SUPRAS_{(oleoresin)}$-NLCs: 0.75±0.15 and 2.92±0.58 μM α-TE,respectively.

Most of the of values previously reported for the antioxidant activity of astaxanthin extracted from *Haematococus pluvialis* using the ABT assay are expressed as mmol Trolox/g extract (and not α-Tocopherol equivalent/g extract) and are quite similar. Thus, they are0.18 ± 0.01 for dimethyl sulfoxide extracts (Regnier et al., 2015), from 0.134 ± 0.004 to 0.196 ± 0.003 and 0.232 ± 0.004 to 0.267 ± 0.005 for PLE extracts (extraction temperature 50-200 ºC) using hexane and ethanol, respectively (Jaime et al., 2010), and 0.243 for SFE extracts using 13% ethanol as cosolvent (Reyes et al., 2014). Very few data are available in the literature concerning α-Tocopherol equivalent antioxidant activity. In this context, the α-TEAC value (0.8) reported by Müller et al. for acetone extracts from *Haematococcus pluvialis* (Müller, Fröhlich, &Böhm, 2011) is quite similar to that obtained for $SUPRAS_{(oleoresin)}$ (0.75 ± 0.15) and lower than the value obtained for $SUPRAS_{(oleoresin)}$-NLC (2.92 ± 0.58).

On the other hand, the ORAC value obtained for dimethyl sulfoxide extracts was 8.1 ± 1.2 μM TE (Regnier et al., 2015) while those obtained from $SUPRAS_{(oleoresin)}$ and $SUPRAS_{(oleoresin)}$-NLC were 15.1 ± 2.7 μM TE and 20.6 ± 3.9 μM TE, respectively (when expressed as mmol Trolox/g extract : 0.14±0.03 and 0.20±0.04). Hossain et al.

have reported similar ORAC results (mmol TE/g sample) from 0.08 to 0.11 (Hossain et al., 2017).

The results here obtained for antioxidant activity of astaxanthin show that, as comparable, they are in the same range for $SUPRAS_{(oleoresin)}$ and increase for $SUPRAS_{(oleoresin)}$-NLCs. This fact confirms the preservation of the astaxanthin activity after extraction in SUPRAS, since both tests show that no activity is lost during the encapsulation procedure thus confirming one of the hypotheses of this work, i.e. that SUPRAS-NLCs are an excellent and novel approach to simultaneously extract and encapsulate astaxanthin from *H. pluvialis*.

In vitro inhibition of cellular reactive oxygen species by SUPRAS-NLCs

An excess of reactive oxygen species (ROS) is involved in the pathogenesis of several diseases. In that sense, astaxanthin has been shown to exhibit potent antioxidant, anti-inflammatory and cardio-protective activities (Fassett & Coombes, 2011). Several experimental data support the protective role of astaxanthin on endothelial cells under oxidative stress; (Zuluaga, Barzegari, Letourneur, Gueguen, & Pavon-Djavid, 2017). Here, we measured the capacity of $SUPRAS_{(oleoresin)}$-NLCs to protect endothelial human cells under stress. Dihydroethidium (DHE), a lipophilic cell permeable fluorogenic dye, was used to measure intracellular superoxide radicals ($O_2^{\cdot-}$). When it undergoes oxidation, DHE intercalates with the DNA giving red fluorescence. Human umbilical vein endothelial cells (HUVEC) were cultured in presence of $SUPRAS_{(oleoresin)}$-NLCs, $SUPRAS_{(blank)}$-NLCs or standard antioxidant (NAC) during 24, 48 and 72h. Then, cellular stress was initiated by addition of antimycin A, which induces superoxide radicals ($O_2^{\cdot-}$) production through mitochondrial depolarization, thus blocking the electron transport chain (Dikalov& Harrison, 2012). A significant increase in the intracellular fluorescence emission was recorded after addition of antimycin A compared to the control cells under basal conditions ($p<0.05$) (Figure 3.D). The $SUPRAS_{(oleoresin)}$-NLCsprotection effect against ROS excess was clear, since a reduction of the fluorescence emission tothe level measured for basal conditions and NAC was achieved ($p>0.05$). When endothelial cells were preincubated 48 and 72h with $SUPRAS_{(oleoresin)}$-NLCs, the intracellular ROS accumulation was significantly diminished even compared to NAC. A significant difference was also observed between $SUPRAS_{(oleoresin)}$-NLCs and $SUPRAS_{(blank)}$-NLCs($p<0.05$), which highlights the role of astaxanthin in ROS

inhibition. ROS scavenging efficiency (*RSE, %)* values weresignificantly higher for $SUPRAS_{(oleoresin)}$-NLCs (36.8; 53.8; 67.2) than forNAC (36.1;33.3 45.3) ($p < 0.05$). These results show that $SUPRAS_{(oleoresin)}$-NLCs preserve astaxanthin antioxidant activity in *in vitro* conditions, and that they are capable of blocking excess intracellular ROS in endothelial cells under oxidative stress.

Time stability of SUPRAS-NLCs

One of the major problems of the use of NLCs for the encapsulation of carotenoids is the low stability they present after encapsulation. These NLCs usually lose all their properties in a short period of time (Tamjidi, Shahedi, Varshosaz, &Nasirpour, 2014). One of the main hypotheses of this work is that SUPRAS may influence not only on the extraction and encapsulation but also on the conservation and stability over time of NLCs. In order to prove this hypothesis, we determine encapsulation efficiencies, sizes and zeta potentials, 0, 30, 45, 60 and 180 days after synthetizing the $SUPRAS_{(oleoresin)}$-NLCs. Results showed that these NLCs present a high stability, with an encapsulation efficiency of 71 ± 3% with a null variation over time. Furthermore, sizes and zeta potentials for $SUPRAS_{(oleoresin)}$-NLCs do not present a great variation in time (Figure 4.A). Cryo-SEM microphotographs (Fig. 4 inset) confirm that these NLCs keep the spherical morphology over the whole period without increasing their size. The high stability of $SUPRAS_{(oleoresin)}$-NLCs proved by these results highlights the critical role of SUPRAS encapsulation for astaxanthin stabilization. Besides, $SUPRAS_{(blank)}$-NLCs (Figure 4.B) showed an equivalent stability.

The study of the antioxidant capacity as a function of time was investigated using the α-TEAC method since the lineal interval for the response of $SUPRAS_{(oleoresin)}$-NLCs was much wider for α-TEAC compared to ORAC (e.g. see the results obtained for ORAC and α-TEAC in figures 3B and 3C, respectively). So the detection of both low and high variations of the antioxidant capacity of $SUPRAS_{(oleoresin)}$-NLCs with time should be more precise using α-TEAC. The results obtained showed that, after 60 days, the antioxidant activity of $SUPRAS_{(oleoresin)}$-NLCs was similar to the initial activity (2.53±0.50 and 2.91±0.58 μM α-TE, respectively). These results show that the antioxidant activity of $SUPRAS_{(oleoresin)}$-NLCs is very stable over time, especially when compared to actual encapsulation methodologies, which confirms one of the main hypotheses of this work.

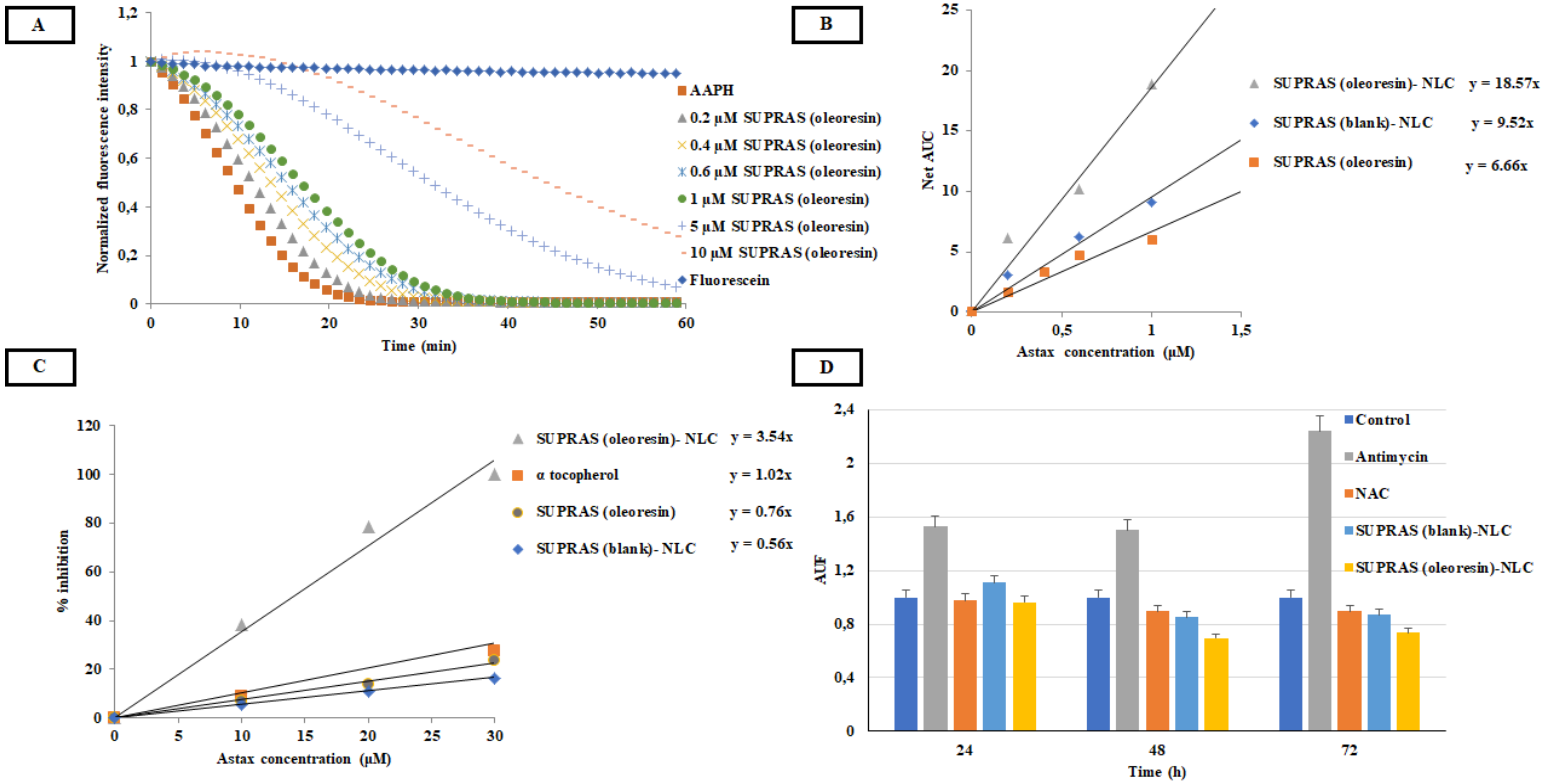

Figure 3. Results from ORAC test (A & B), ABTS assay (C) and DHE test (D) for SUPRAS-NLCs

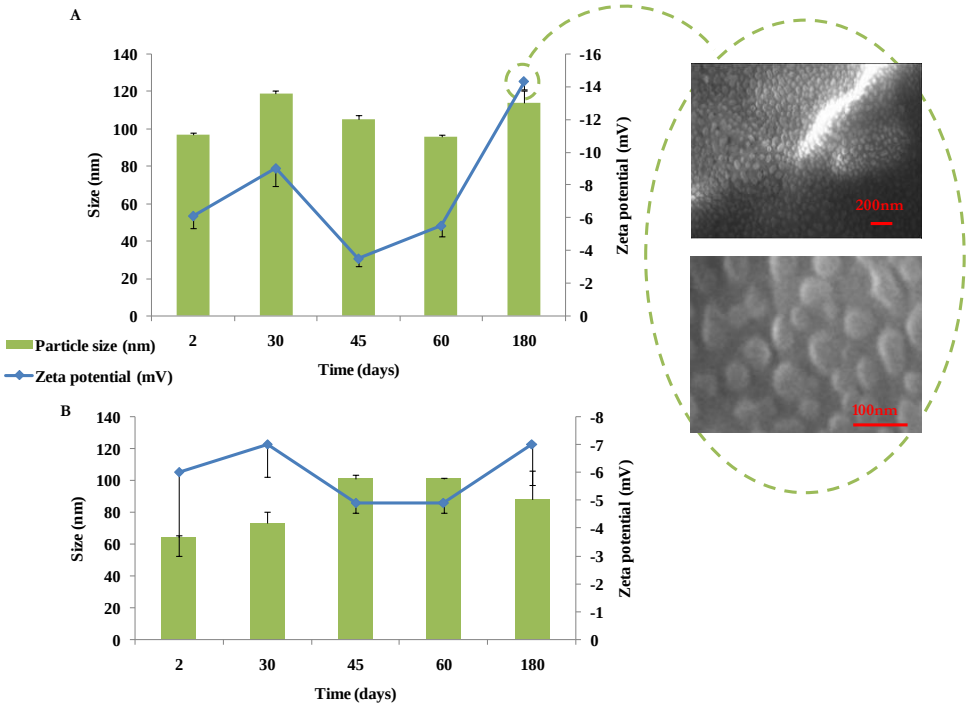

Figure 4. SUPRAS$_{(oleoresin)}$-NLCs stability in terms of size and zeta potential over 180 days for SUPRAS$_{(oleoresin)}$-NLCs (A) and SUPRAS$_{(blank)}$-NLCs (B). Inset: cryoSEM microphotographs for SUPRAS$_{(oleoresin)}$-NLCs 180 days after synthesis

CONCLUSIONS

A novel green chemical process for extraction, encapsulation and stabilization of astaxanthin from *Haematococcus pluvialis* based on the direct combination of SUPRAS with NLCs has been successfully achieved. The SUPRAS$_{(oleoresin)}$-NLCs obtained exhibited high encapsulation efficiency (71±4) and load (24±1),with a small diameter (~100 nm) and a low PDI (~0.3). It has been demonstrated the fundamental role of SUPRAS in obtaining high extraction yields, proper NLCs and even an unprecedented time stability, being stable for at least 180 days at 4ºC. Additionally, SUPRAS$_{(oleoresin)}$-NLCs were able to effectively preserve the astaxanthin antioxidant activity. Their biochemical scavenging capacity was higher than that of standards Trolox (20.6± 3.9 μM TE) and α-Tocopherol (2.92±0.58 μM α-TE). Furthermore, they showed *in vitro* capacity to protect human endothelial cells from ROS. In conclusion, SUPRAS$_{(oleoresin)}$-NLCs are a new, cost-effective, and sustainable methodology for extraction and preservation of natural astaxanthin that may be useful for a wide range of food and medical applications. Moreover, it is our opinion that this novel combination of

SUPRAS and NLCs, as an extraction-encapsulation-stabilization vector, could be extended to several other compounds and matrices of interest in food chemistry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Conclusiones

La investigación propuesta en esta Tesis Doctoral se ha dirigido al desarrollo, caracterización y aplicación en procesos de extracción de nuevos disolventes nanoestructurados producidos a partir de sustancias anfifílicas utilizando el autoensamblaje como ruta de síntesis y el agua como agente coacervante. De esta manera, se ha pretendido extender su uso a nuevos formatos de extracción y a procesos que hasta ahora se veían limitados por su alto coste o por la dificultad de mantener las condiciones de autoensamblaje.

Además, la investigación propuesta en esta tesis ha buscado aumentar los conocimientos sobre la síntesis a medida, composición y estructura de los disolventes supramoleculares, desarrollando disolventes programados para cumplir funciones específicas. Una de las prioridades en las cuales esta investigación se ha enfocado activamente ha sido la consolidación del uso de estos disolventes en extracciones analíticas e industriales y en áreas y matrices hasta ahora inexploradas.

Por lo tanto, la investigación desarrollada en esta Tesis Doctoral ha supuesto un avance en el diseño de nuevos disolventes que cumplen con los principios de la química verde y que tienen la capacidad de mejorar, y permiten el desarrollo de aplicaciones innovadoras, en procesos de extracción.

En particular, debe destacarse el avance en la aplicación de SUPRAS volátiles con propiedades de acceso restringido (RAM-VOL-SUPRAS), el desarrollo y aplicación de SUPRAS con incorporación cuantitativa del anfifilo (SUPRAS basados en oligómeros), SUPRAS de alta estabilidad térmica (HTS-SUPRAS) y la combinación de SUPRAS con sistemas lipídicos nanoestructurados (SUPRAS-NLCs).

Capítulo I: Disolventes supramoleculares constituidos por compuestos anfifílicos volátiles para reducción de los efectos matriz originados por fosfolípidos en LC-MS/MS

Los resultados recogidos en este capítulo han demostrado que los SUPRAS volátiles con propiedades de acceso restringido constituyen una herramienta adecuada para eliminar proteínas y fosfolípidos de muestras de orina y, por lo tanto, para eliminar el efecto de matriz causado por estos componentes en LC-MS/MS. El enfoque aquí planteado, basado en la precipitación de ambos componentes endógenos, tiene el potencial necesario para poder constituirse como una herramienta genérica por el tratamiento de muestras biológicas.

Dos de los beneficios más importantes del procedimiento desarrollado son su simplicidad desde el punto de visto práctico y su elevada eficiencia en la eliminación de interferentes y extracción del analito de interés. Así, solo son necesarios bajos volúmenes de los ingredientes (83 μL de hexanol y 150 μL de THF por muestra de orina) para formar el SUPRAS, precipitar las proteínas y extraer bisfenol A. Finalmente, cabe enfatizar que el tratamiento de muestra propuesto solo requiere de instrumentación convencional, su bajo coste y que varias muestras pueden ser procesadas simultáneamente. Por otra parte, hay que recalcar que el método propuesto no es válido para compuestos volátiles, sin embargo, los compuestos analizados usualmente en bioanálisis mediante LC-MS son comúnmente polares y no volátiles.

Capítulo II: Disolventes supramoleculares constituidos por tensioactivos oligoméricos para reducir las pérdidas de tensioactivo en la disolución de equilibrio

En las investigaciones desarrolladas en este capítulo se ha sintetizado un nuevo SUPRAS basado en un tensioactivo oligomérico que se incorpora cuantitativamente al disolvente supramolecular. Esta característica puede ser de gran interés en campos donde la aplicación del SUPRAS está limitada por la presencia del anfifilo en la disolución de equilibrio, lo que puede conllevar problemas medioambientales o de salud, como por ejemplo la descontaminación de agua o la manufactura de alimentos funcionales.

Se seleccionaron dos disolventes orgánicos miscibles con agua de diferentes características fisicoquímicas, etanol (prótico) y THF (aprótico) y se estudió su efecto en la síntesis del SUPRAS, evaluando su formación, composición y volumen. Con respecto a la región de formación del SUPRAS en el diagrama de fases, fue más amplia cuando se utilizó THF, comportamiento que puede ser atribuido a la considerable diferencia en los valores del parámetro de solubilidad de Hildebrand para los disolventes evaluados (5 para etanol y 15 para THF). Las composiciones de ambos SUPRAS también presentaron diferencias respecto al contenido de agua incorporada, mayor cuando se empleó etanol, mientras que el oligómero se incorporó de manera similar en ambos casos. Finalmente, respecto al volumen del SUPRAS formado, fue mayor cuando se utilizó THF como disolvente. Basándonos en estos resultados, se propone el empleo de

THF para la síntesis de SUPRAS a partir del tensioactivo oligomérico, con agua como agente coacervante, dado que muestra una mayor versatilidad.

Capítulo III: Disolventes supramoleculares térmicamente estables para aplicación en cromatografía de gases con espacio de cabeza

El SUPRAS sintetizado a partir de disoluciones de un tensioactivo oligomérico en tetraglima ha permitido hacer compatible los extractos supramoleculares con HS-GC-MS. Esta compatibilidad se ha comprobado mediante la determinación de disolventes residuales, que presentan un amplio intervalo de puntos de ebullición, en fármacos. Comparada con otras estrategias habituales como la utilización de disolventes de alto punto de ebullición o líquidos iónicos, puede concluirse que la extracción con SUPRAS ofrece ventajas en términos de solubilidad, estabilidad térmica, facilidad de síntesis y coste.

Capítulo IV: Disolventes supramoleculares multifuncionales para combinar la extracción y encapsulación de componentes bioactivos lipofílicos

En las investigaciones descritas en este capítulo se ha desarrollado un proceso químico sostenible para la extracción, encapsulación y estabilización de astaxantina a partir de *Hematococcus pluvialis* basada en la combinación directa de SUPRAS y portadores lipídicos nanoestructurados (NLCs). Se ha demostrado el rol fundamental de los SUPRAS en la obtención de altos rendimientos de extracción y estabilización de astaxantina y en la síntesis de las NLCs. Además, las nanopartículas sintetizadas permitieron preservar el poder antioxidante de la astaxantina. En conclusión, los SUPRAS-NLCs constituyen una metodología nueva, económica y sostenible para la extracción y conservación de astaxantina natural que puede resultar de utilidad para un gran número de aplicaciones tanto médicas como alimentarias y que puede extenderse a la extracción y encapsulamiento de otras sustancias bioactivas.

Conclusions

The research proposed in this Doctoral Thesis has been aimed at the development, characterization and application in extraction processes of new nanostructured solvents produced from amphiphilic substances using self-assembly as a synthetic route and water as a coacervating agent. In this way, it has been intended to extend their use to new extraction formats and to processes that until now have been limited by the high cost or by the difficulty of maintaining self-assembly conditions.

In addition, the research proposed in this thesis has sought to increase the previous knowledge regarding the tailoring, composition and structure of supramolecular solvents, by developing solvents programmed to fulfill specific functions. Therefore, the progress in consolidating their use in analytical and industrial extractions in areas and matrices hitherto unexplored has been one of the priorities in which this research has actively focused on.

Thus, the research developed in this Doctoral Thesis has led to progress in the design of novel solvents fulfilling the principles of green chemistry and having the capacity to improve, and allowing the development of innovative applications, in extraction processes.

In particular, it should be highlighted the progress in the application of volatile SUPRAS with restrictive access properties (RAM-VOL-SUPRAS), the development and application of amphiphile's quantitative incorporation SUPRAS (oligomer-based SUPRAS), high thermal stability SUPRAS (HTS-SUPRAS), and the combination of SUPRAS and nanostructured lipid carriers (SUPRAS-NLCs).

Further specific conclusions are specified below:

Chapter I: Volatile amphiphile-based supramolecular solvents for reducing phospholipid-based matrix effects in LC-MS/MS

The results obtained in this chapter have demonstrated that volatile SUPRAS with restricted access properties are a suitable tool for removing proteins and phospholipids from urine samples and, therefore, to avoid the matrix effect caused by these components in LC-MS/MS. The approach proposed here, based on the precipitation of both endogenous components, has the potential to be considered as a generic tool for the treatment of biological samples.

Two of the most important advantages of the procedure here developed are its simplicity from the practical point of view and its high efficiency in the removal of interferences and in the extraction of the compound of interest. Thus, just low volumes of the ingredients are required (83 μL of hexanol and 150 μL of THF per urine sample) in order to obtain the SUPRAS, precipitate the proteins and extract bisphenol A. Finally, it should be highlighted that this sample treatment only requires of conventional instrumentation, its low cost, and the fact that several samples can be simultaneously processed. On the other hand, it should be noted that the method is not suitable for volatile compounds; however, the compounds usually analyzed by LC-MS in bioanalysis are usually polar and non-volatile.

Chapter II: Supramolecular solvents formed by oligomeric surfactants to reduce surfactant losses in the equilibrium solution

The research developed in this chapter consists of the synthesis of a novel SUPRAS based on an oligomeric surfactant that is quantitatively incorporated into the supramolecular solvent. This feature can be of great interest in fields where the application of SUPRAS is limited by the occurrence of the amphiphile in the equilibrium solution that may lead to environmental or health problems, such as water purification or the manufacture of functional foods.

Two water miscible organic solvents with different physicochemical properties were selected, namely ethanol (protic) and THF (aprotic), in order to study aspects related to the formation, the composition and the volume of the SUPRAS obtained. Regarding the SUPRAS formation region in the phase diagram, it was broader when THF was used as organic solvent, behavior that can be attributed to the difference in the values of the Hildebrand solubility parameter for the solvents evaluated (5 for ethanol and 15 for THF). The composition of both SUPRAS also showed differences with respect to the water content, which was higher for ethanol, while the oligomer was incorporated in a similar proportion, ca. 100%, for both solvents. Finally, the volume of SUPRAS obtained was higher when THF was used as organic solvent. In conclusion, the use of THF is prompted for the synthesis of SUPRAS based on the oligomeric surfactant proposed, with water as coacervating agent, since it shows a greater versatility.

Chapter III: High thermally stable supramolecular solvents applicable to headspace gas chromatography

The novel HTS-SUPRAS, synthesized from solutions of an oligomeric surfactant in tetraglyme, has allowed to obtain supramolecular extracts compatible with HS-GC-MS. This compatibility has been proven by the determination of residual solvents, which have a wide range of boiling points, in drugs. Compared with other common strategies such as the use of high-boiling point solvents or ionic liquids, it can be concluded that HTS-SUPRAS extraction offers great advantages in terms of solubility, thermal stability, ease of synthesis and cost.

Chapter IV: Multifunctional supramolecular solvents for the combination of the extraction and encapsulation of lipophilic bioactive components

The research in this chapter describes the development of a sustainable chemical process for the extraction, encapsulation and stabilization of astaxanthin from *Hematococcus pluvialis*, based on the direct combination of SUPRAS and nanostructured lipid carriers (NLCs). The role of SUPRAS in obtaining high yields for the extraction and stabilization of astaxanthin and in the synthesis of NLCs has been demonstrated. Additionally, the nanoparticles synthesized allowed to preserve the antioxidant properties of astaxanthin. In conclusion, SUPRAS-NLCs are a new, cost-effective and sustainable methodology for the extraction and preservation of natural astaxanthin that could be useful for several medical and food applications and that could be extended to the extraction and encapsulation of other bioactive substances.

Apéndice A

Publicaciones científicas derivadas de la Tesis Doctoral

1. *"Volatile restricted access supramolecular solvents: Synthesis, characterization and application in biological analysis"*

   J.A. Salatti-Dorado, N. Caballero-Casero, M.D. Sicilia, L. Lunar, Soledad. Rubio.

   Anal. Chim. Acta 9 (50) 71-79 (**2017**)

2. *"A High Thermally Stable Oligomer-Based Supramolecular Solvent for Universal Headspace Gas Chromatography: Proof-of-Principle Determination of Residual Solvents in Drugs".*

   J.A. Salatti-Dorado, S. González-Rubio, D. García-Gómez, Rafael Lucena, Soledad Cárdenas, Soledad. Rubio.

   Anal. Chim. Acta. In press. https://doi.org/10.1016/j.aca.2018.09.023

3. *"Multifunctional green supramolecular solvents for cost-effective production of highly stable astaxanthin-rich formulations from Haematococcus pluvialis"*

   J.A. Salatti-Dorado, V. Rodriguez-Ruiz, D. García-Gómez, V. Gueguen, S. Rubio, G. Pavon-Djavid.

   Food Chemistry revision.

4. *"Astaxanthin-loaded nanostructured lipid carriers for preservation of antioxidant activity"*

   Rodriguez-Ruiz V, Salatti-Dorado JA, Barzegari A, Nicolas-Boluda A, Houaoui A, Caballo C, Caballero-Casero N., Sicilia MD, Bastias Venegas J, Pauthe E, Omidi Y, Letourneur D, Rubio S, Gueguen V, Pavon-Djavid G

   Molecules 23(10) 2601(**2018**)**.**

Apéndice B

ORAL NACIONAL

UN NUEVO DISOLVENTE SUPRAMOLECULAR BASADO EN MICELAS OLIGOMÉRICAS: SÍNTESIS Y CARACTERIZACIÓN.

Congreso: IV Reunión de Jóvenes Investigadores en Coloides e Interfases

Córdoba, del 7 al 9 de febrero de 2018

IV reunión de Jóvenes Investigadores en Coloides e Interfases

Córdoba, 7-9 Febrero 2018

Universidad de Córdoba

JICI-IV

Libro de resúmenes

IV Reunión de Jóvenes Investigadores en Coloides e Interfases (JICI IV) Córdoba, 2018

Presentaciones orales **O-43**

UN NUEVO DISOLVENTE SUPRAMOLECULAR BASADO EN MICELAS OLIGOMÉRICAS: SÍNTESIS Y CARACTERIZACIÓN.

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SUPRAS (disolvente supramolecular) es un término relativamente reciente que hace referencia a la fase rica en tensioactivos (coacervado) separada de disoluciones coloidales mediante procesos de autoensamblaje. Este término enfatiza cómo los anfífilos forman estructuras supramoleculares autoorganizadas en la fase líquida separada, siendo esta la característica más distintiva en comparación con los disolventes moleculares y los líquidos iónicos. Una de las mayores desventajas en el uso de SUPRASs en química analítica es la pérdida de anfífilo que se produce en el tratamiento de muestras de elevado volumen, ya que el anfífilo se encuentra en equilibrio con la fase acuosa -a la concentración micelar crítica (CMC)- redundando este hecho en recuperaciones no cuantitativas. Por otro lado, la mayoría de los SUPRAS son incompatibles con cromatografía de gases, dada la elevada concentración de tensioactivo que se volatiliza. En este trabajo se sintetizaron y caracterizaron SUPRASs basados en oligómeros de ácido undecenoico para los que se ha propuesto en el pasado que su CMC es nula o, al menos, despreciable y que presentan elevado punto de ebullición.

Los SUPRASs aquí estudiados se sintetizaron añadiendo agua a disoluciones de oligómeros de ácido undecenoico (P.UDA) en tetrahidrofurano (THF). El agua promovió el autoensamblaje del oligómero y la separación de fases líquidas (Fig. 1). Se obtuvo así el respectivo diagrama de fases a partir de mezclas ternarias de P.UDA/THF/agua. Adicionalmente, se investigó la influencia de la temperatura y de la concentración de sales en estos límites y se determinó la ecuación empírica que relaciona el volumen de SUPRAS obtenido con la cantidad de anfífilo y agua utilizados en la síntesis. Finalmente, se evaluó la composición del SUPRAS bajo diferentes condiciones sintéticas y la organización nanoestructural mediante la técnica Cryo-SEM (Fig. 1).

Los estudios realizados demostraron que la composición global del disolvente y el tamaño de las gotitas de coacervado que lo forman pueden modificarse controlando el ambiente en el que se produce el autoensamblaje. Así, los SUPRASs caracterizados en este trabajo son altamente adaptativos, pudiendo revertirse sus características mediante modificación del modificando el entorno. En todo caso, el autoensamblaje espontáneo de estos disolventes siguió rutas predecibles, y su composición y volumen pueden preverse con precisión a partir de ecuaciones empíricas. Las propiedades descritas que presenta este tipo de SUPRAS los hacen sumamente atractivos para la extracción de analitos mediante cromatografía de gases acoplada a espacio de cabeza (HS-GC). Gracias a la incorporación cuantitativa del anfífilo, las extracciones resultan altamente eficientes en un gran intervalo de condiciones iniciales y, a diferencia de otros SUPRAS caracterizados anteriormente, su baja volatilidad los hace sumamente válidos para aplicaciones analíticas en dicha técnica de separación presentando cromatogramas HS-GC libres de interferencias.

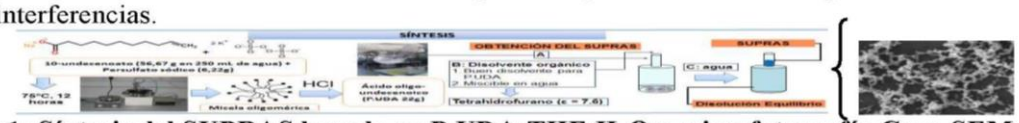

Fig.1: Síntesis del SUPRAS basado en P.UDA:THF:H_2O y microfotografía Cryo-SEM

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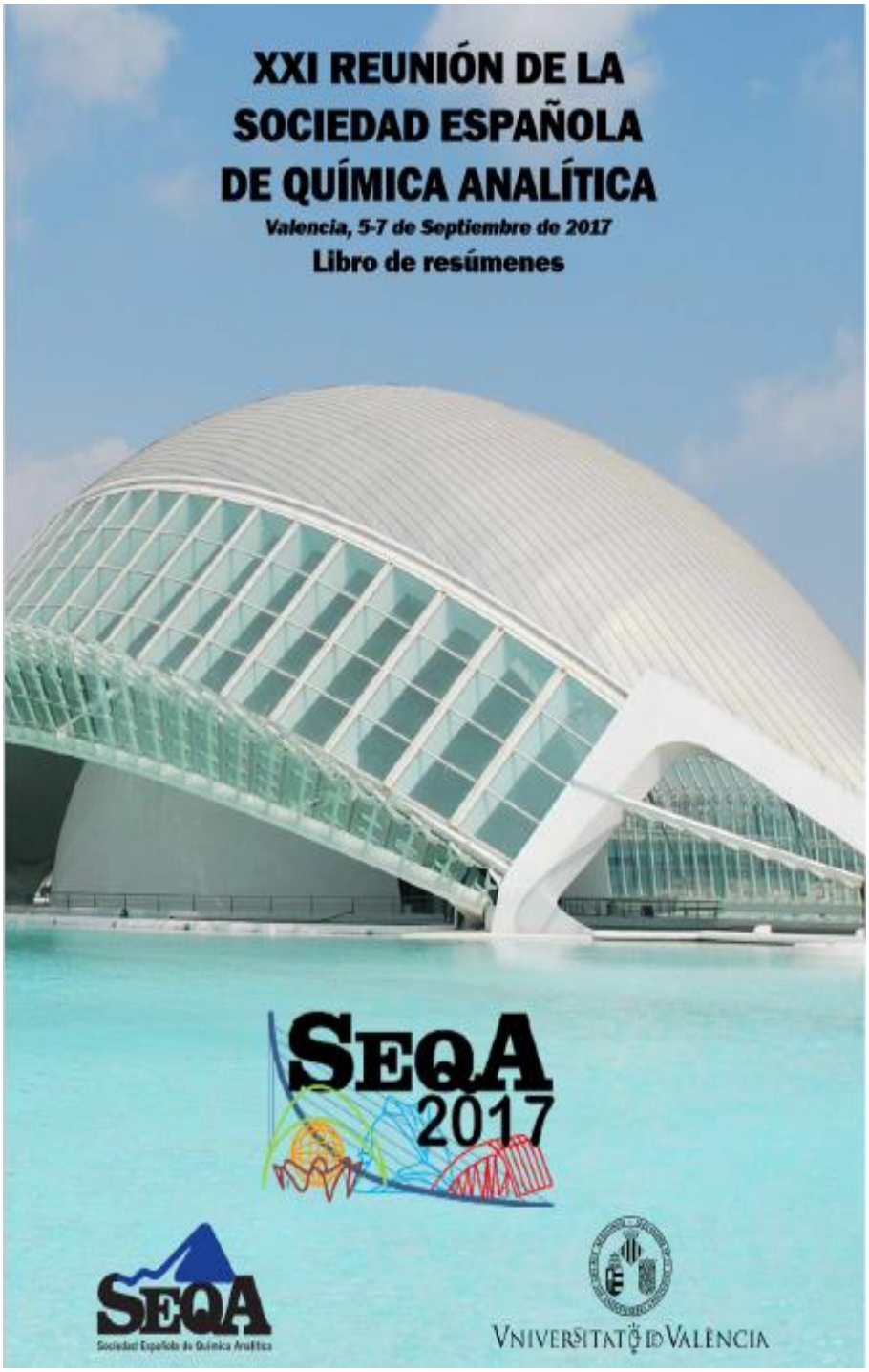
ORAL NACIONAL

MODELADO DE PROPIEDADES DE DISOLVENTES SUPRAMOLECULARES PARA PROCESOS DE EXTRACCIÓN ANALÍTICA

Congreso: XXI Reunión de la Sociedad Española de Química Analítica

Valencia, del 5 al 7 de septiembre de 2017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OR-21

MODELADO DE PROPIEDADES DE DISOLVENTES SUPRAMOLECULARES PARA PROCESOS DE EXTRACCIÓN ANALÍTICA

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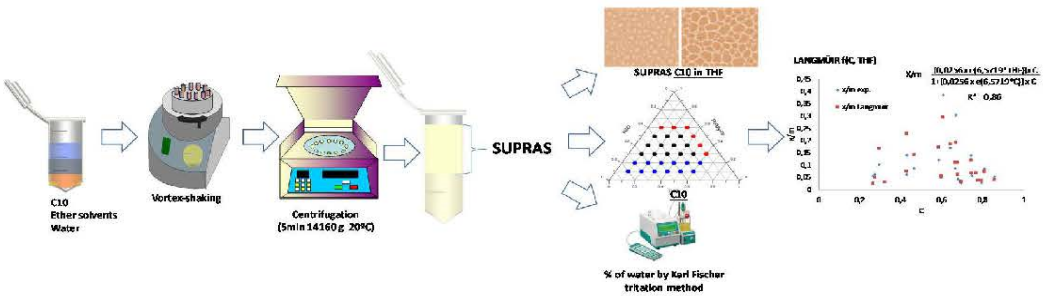
Los disolventes supramoleculares (SUPRAS) pueden definirse como fases ricas en tensioactivo con organización a dos niveles, nano y microscópico. Se obtienen a partir de disoluciones acuosas o hidro-orgánicas de tensioactivo mediante procesos de autoensamblaje y coacervación. Su aplicación en procesos de extracción analítica es un área en expansión dada la elevada eficacia de extracción proporcionada por los SUPRAS para compuestos en un muy amplio intervalo de polaridad y peso molecular, y en muestras de diferente naturaleza y complejidad.

Una de las características más relevantes de los SUPRAS es la posibilidad de diseñar disolventes con propiedades programadas para que cumplan funciones específicas. Esta característica deriva de la reversibilidad/reorganización de las estructuras supramoleculares que lo integran en respuesta a modificaciones ambientales. Así, se han obtenido SUPRAS a partir de alcoholes alquílicos y ácidos carboxílicos en medio THF-agua que muestran propiedades de acceso restringido, lo que ha permitido la integración de la extracción y limpieza de la muestra en una única etapa.

Hasta la fecha, el diseño de SUPRAS se ha basado en ensayos de prueba y error y el estudio de sus propiedades en el ajuste de ecuaciones matemáticas a los resultados experimentales. Dado que el diseño y producción de disolventes con propiedades programadas aumenta notablemente la capacidad de mejorar la selectividad, rendimiento y costes de los procesos de extracción, sería de gran interés el desarrollo de modelos teóricos que permitan predecir las propiedades de los SUPRAS en función de las condiciones ambientales.

Se presenta en esta comunicación un primer modelo teórico, basado en una modificación del modelo clásico de adsorción de Langmuir, para SUPRAS sintetizados a partir de ácidos carboxílicos en medios hidro-orgánicos. Este modelo permite predecir, a partir de la composición inicial del medio hidro-orgánico, la composición acuosa de los agregados hexagonales inversos que constituyen el SUPRAS. Esto permite, por consiguiente, estimar el tamaño de la cavidad acuosa de los mismos, y, como consecuencia, evaluar su capacidad para la exclusión de compuestos en base a su tamaño molecular. Su aplicabilidad se ha comprobado para uno de los tensioactivos más utilizados (ácido decanoico) y para muy diversos disolventes orgánicos de naturaleza etérea (THF, dioxano, dioxolano y mono-, di-, tri-, tetra- y poli-glima). En todos los casos, el ajuste de los datos experimentales al modelo planteado fue muy satisfactorio ($r > 0,9$). Cabe destacar que el desarrollo de este modelo teórico no solo permite predecir la composición del SUPRAS y las propiedades que de ella se derivan, sino que también establece las bases para un conocimiento más profundo de estos sistemas supramoleculares. Por ejemplo, el modelo planteado responde a la ecuación de Van't Hoff, lo que permite la obtención directa de parámetros termodinámicos como la entalpía y la entropía del proceso de autoensamblaje del SUPRAS.



Agradecimientos: Los autores agradecen el apoyo financiero del MINECO (Proyecto CTQ2014-53539-R) y FEDER. J.A.S.D. y D.G.G. agradecen igualmente sus contratos de postgrado (FPU13/03796) y postdoctoral (FJCI-2014-20052), respectivamente.

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PÓSTER, FLASH NACIONAL Y ARTÍCULO BOLETÍN SEQA

SEPTIEMBRE 2017

IMPLEMENTACIÓN DE PERSONAL RESPONSE SYSTEMS EN ASIGNATURAS DE LOS GRADOS DE QUÍMICA Y BIOQUÍMICA

Congreso: III Jornadas sobre estrategias para la innovación de la actividad docente en Química Analítica, carotenoides y herramientas

Valencia, del 5 al 7 de septiembre de 2017


XXI REUNIÓN DE LA
SOCIEDAD ESPAÑOLA
DE QUÍMICA ANALÍTICA
Valencia, 5-7 de Septiembre de 2017
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SEQA
Sociedad Española de Química Analítica
VNIVERSITAT DE VALÈNCIA

P-1

Implementación de Personal Response Systems en asignaturas de los Grados de Química y Bioquímica

J.Á. Salatti-Dorado[a], B. Fresco Cala[a], J. Ríos Gómez[a], M.I. López Martínez[b], J.M.I Fernández Romero[a], F.J. Romero Salguero[b] y Mª.S. Cárdenas Aranzana[a]

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La imagen de la docencia universitaria tradicional se ha visto modificada por los principios que definen el Espacio Europeo de Educación Superior. La enseñanza en la Universidad pretende desarrollar capacidades de autoaprendizaje, de iniciativa y capacidad de visualización de los problemas planteados en cualquiera de sus ámbitos profesionales, así como la habilidad de adoptar soluciones que permitan resolver los problemas que la sociedad les plantee. En este sentido, se requiere que el alumno adquiera las competencias para aplicar los conocimientos adquiridos en las clases magistrales a supuestos prácticos. Por parte del profesor, esto implica una adaptación de su metodología de manera que se incremente el trabajo más personalizado con los alumnos en los grupos de docencia reducidos. Por otra parte, la innovación es un concepto que debe ir incorporándose transversalmente a la docencia, es por ello que cada vez más habitualmente se están usando herramientas que estimulan la participación activa de los alumnos. La cuestión fundamental planteada es si realmente con este tipo de actividades y enseñanza que se dan a los

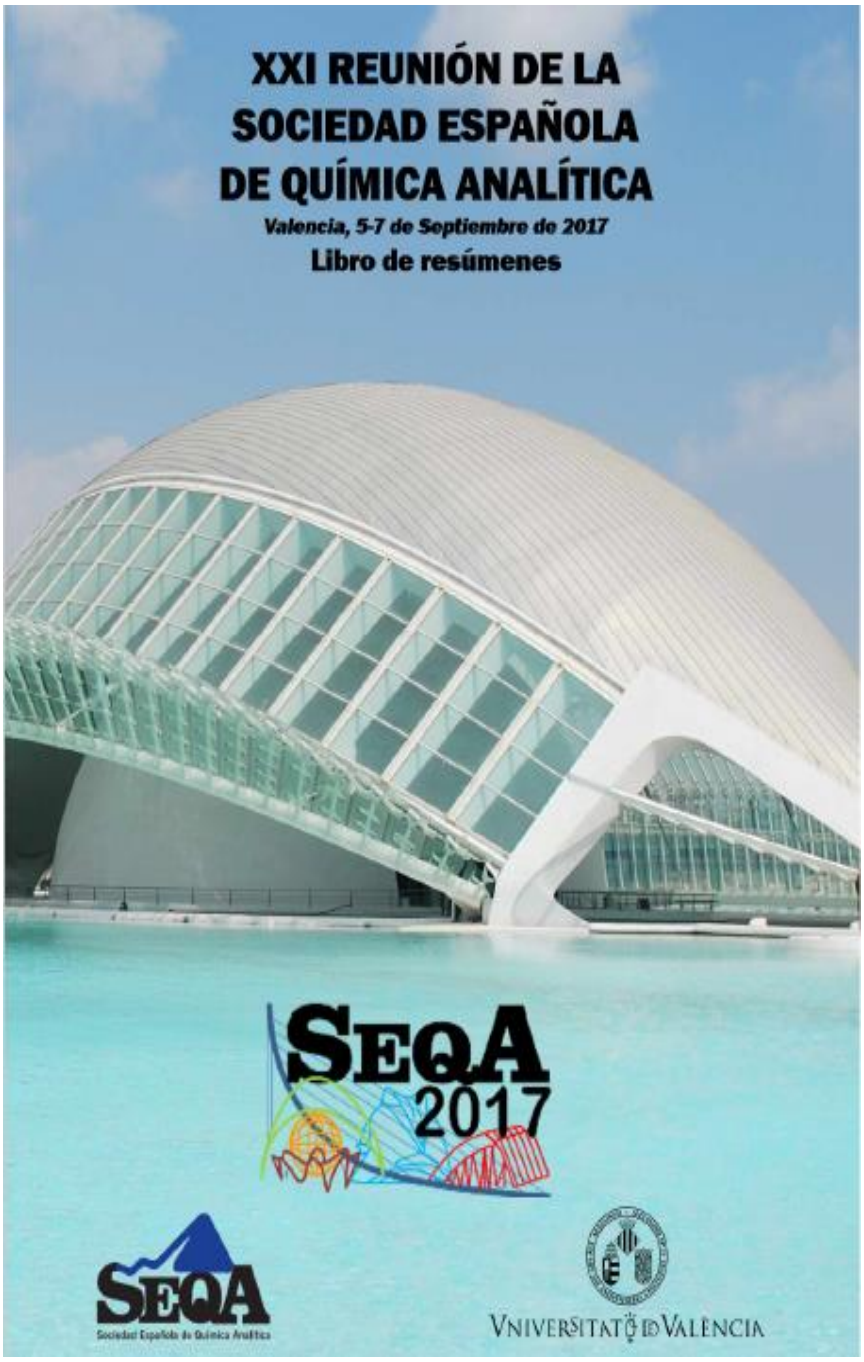
III Jornada sobre estrategias para la innovación de la actividad docente en Química Analítica: contenidos y herramientas. Universitat de València, 5 de septiembre 2017

PÓSTER NACIONAL

MODELADO DE PROPIEDADES DE DISOLVENTES SUPRAMOLECULARES PARA PROCESOS DE EXTRACCIÓN ANALÍTICA

Congreso: XXI Reunión de la Sociedad Española de Química Analítica

Valencia, del 5 al 7 de septiembre de 2017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PO-TM-02

SÍNTESIS Y CARACTERIZACIÓN DE DISOLVENTES SUPRAMOLECULARES CONSTITUIDOS POR AGREGADOS DE MICELAS OLIGOMÉRICAS DE ÁCIDO UNDECENOICO: APLICACIÓN EN PROCESOS EXTRACTIVOS

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SUPRAS (disolvente supramolecular) es un término relativamente reciente que hace referencia a la fase rica en tensioactivos (coacervado) separada de disoluciones coloidales mediante procesos de autoensamblaje. Este término enfatiza cómo los anfifilos forman estructuras supramoleculares autoorganizadas en la fase líquida separada, siendo esta la característica más distintiva en comparación con los disolventes moleculares y los líquidos iónicos. Una de las mayores desventajas en el uso de SUPRASs en química analítica es la pérdida de anfifilo que se produce en el tratamiento de muestras de elevado volumen, ya que el anfifilo se encuentra en equilibrio con la fase acuosa -a la concentración micelar crítica (CMC)- redundando este hecho en recuperaciones no cuantitativas. Por otro lado, la mayoría de los SUPRAS son incompatibles con cromatografía de gases, dada la elevada concentración de tensioactivo que se volatiliza. En este trabajo se sintetizaron y caracterizaron SUPRASs basados en oligómeros de ácido undecenoico para los que se ha propuesto en el pasado que su CMC es nula o, al menos, despreciable y que presentan elevado punto de ebullición.

Los SUPRASs aquí estudiados se sintetizaron añadiendo agua a disoluciones de oligómeros de ácido undecenoico (P.UDA) en tetrahidrofurano (THF). El agua promovió el autoensamblaje del oligómero y la separación de fases líquidas (Fig. 1). Se obtuvo así el respectivo diagrama de fases a partir de mezclas ternarias de P.UDA/THF/agua. Adicionalmente, se investigó la influencia de la temperatura y de la concentración de sales en estos límites y se determinó la ecuación empírica que relaciona el volumen de SUPRAS obtenido con la cantidad de anfifilo y agua utilizados en la síntesis. Finalmente, se evaluó la composición del SUPRAS bajo diferentes condiciones sintéticas y la organización nanoestructural mediante la técnica Cryo-SEM (Fig. 1).

Los estudios realizados demostraron que la composición global del disolvente y el tamaño de las gotitas de coacervado que lo forman pueden modificarse controlando el ambiente en el que se produce el autoensamblaje. Así, los SUPRASs caracterizados en este trabajo son altamente adaptativos, pudiendo revertirse sus características mediante modificación del modificando el entorno. En todo caso, el autoensamblaje espontáneo de estos disolventes siguió rutas predecibles, y su composición y volumen pueden preverse con precisión a partir de ecuaciones empíricas. Las propiedades descritas que presenta este tipo de SUPRAS los hacen sumamente atractivos para la extracción de analitos mediante cromatografía de gases acoplada a espacio de cabeza: gracias a la incorporación cuantitativa del anfifilo las extracciones resultan altamente eficientes en un gran intervalo de condiciones iniciales y, a diferencia de otros SUPRAS caracterizados anteriormente, su baja volatilidad resulta en cromatogramas HS-GC libres de interferencias generadas por el propio SUPRAS.

Fig.1: Síntesis del SUPRAS basado en P.UDA:THF:H_2O y microfotografía Cryo-SEM

Agradecimientos: Los autores agradecen el apoyo financiero del MINECO (Proyecto CTQ2014-53539-R) y FEDER. J.A.S.D. y D.G.G. reconoce al MINECO por una beca de postgrado (FPU13/03796) y postdoctoral (FJCI-2014-20052), respectivamente.

PÓSTER NACIONAL

SÍNTESIS Y CARACTERIZACIÓN DE DISOLVENTES SUPRAMOLECULARES CONSTITUIDOS POR AGREGADOS ESTABLES DE MICELAS OLIGOMÉRICAS DE ÁCIDO UNDECENOICO

Congreso: Sexta edición de encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces Reunión de la Sociedad Española de Química Analítica

Córdoba, del 25 al 26 de enero de 2017

Sexta Edición del Encuentro sobre Nanociencia y Nanotecnología

de Investigadores y Tecnólogos Andaluces

VI Workshop

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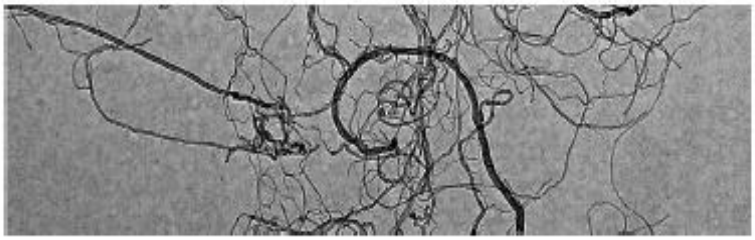

LIBRO DE RESÚMENES

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SÍNTESIS Y CARACTERIZACIÓN DE DISOLVENTES SUPRAMOLECULARES CONSTITUIDOS POR AGREGADOS DE MICELAS OLIGOMÉRICAS DE ÁCIDO UNDECENOICO

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En los últimos años, los procesos de autoensamblaje están dando acceso a materiales supramoleculares funcionales avanzados y proporcionando un enfoque original a la nanociencia y a la nanotecnología. Sin embargo, dicho poder permanece virtualmente inexplorado en lo que se refiere a la producción de disolventes para uso específico, a pesar de que el autoensamblaje ya ha demostrado ser una estrategia a tener en cuenta para la síntesis de fases líquidas ordenadas a partir de anfifilos.

SUPRAS (disolvente supramolecular) es un término relativamente reciente que hace referencia a la fase rica en tensioactivos (coacervado) separada de disoluciones coloidales mediante procesos de autoensamblaje. Este término enfatiza como los anfifilos forman estructuras supramoleculares autoorganizadas en la fase líquida separada, siendo esta la característica más distintiva en comparación con los disolventes moleculares y los líquidos iónicos. Una de las mayores desventajas en el uso de SUPRASs en química analítica es la pérdida de anfifilo que se produce en el tratamiento de muestras de elevado volumen, ya que el anfifilo se encuentra en equilibrio con la fase acuosa -a la concentración micelar crítica (CMC)- redundando este hecho en recuperaciones no cuantitativas. En este trabajo se sintetizaron y caracterizaron SUPRASs basados en oligómeros de ácido undecenoico para los que se ha propuesto en el pasado que su CMC es nula o, al menos, despreciable.

Los SUPRASs aquí estudiados fueron sintetizados añadiendo agua a disoluciones de oligómeros de ácido undecenoico en tetrahidrofurano (THF) o etanol. El agua promovió el autoensamblaje del oligómero y provocó la separación de fases líquidas. Se obtuvieron así los respectivos diagramas de fases a partir de mezclas ternarias de ácido undecenoico/THF/agua y ácido undecenoico/etanol/agua, limitándose la región donde se produjo la separación de fases, es decir, la región de formación de SUPRAS. Adicionalmente, se investigó la influencia de la temperatura y de la concentración de sales en estos límites y se determinó la ecuación empírica que liga el volumen de SUPRAS obtenido con la cantidad de anfifilo y agua utilizados en la síntesis. Finalmente, se evaluó la composición del SUPRAS bajo diferentes condiciones sintéticas y la organización nanoestructural mediante las técnicas TEM y SEM.

Los estudios realizados demostraron que la composición global del disolvente y el tamaño de las gotitas de coacervado que lo forman pueden adaptarse controlando el ambiente en el que se produce el autoensamblaje. Son así los SUPRASs caracterizados en este trabajo altamente adaptativos, pudiendo ser revertidas las características anteriores modificando el entorno. En todo caso, el autoensamblaje espontáneo de estos disolventes siguió rutas predecibles, y su composición y volumen pueden preverse con precisión a partir de ecuaciones empíricas. Adicionalmente, y a diferencia de otros SUPRASs caracterizados anteriormente, se encontró que la incorporación del anfifilo al SUPRAS es cuantitativa, lo que sugiere que podría aplicarse al análisis de muestras de elevado volumen dando lugar a recuperaciones cuantitativas.

Agradecimientos: Los autores agradecen el apoyo financiero del MINECO (Proyecto CTQ2014-53539-R) y FEDER. J.A.S.D. y D.G.G. reconoce al MINECO por una beca de postgrado (FPU13/03796) y postdoctoral (FJCI-2014-20052), respectivamente.

ORAL INTERNACIONAL

ENVIRONMENT-RESPONSIVE SUPRAMOLECULAR SOLVENTS BASED ON THE SELF-ASSEMBLY OF UNDECENOIC ACID POLYMERS: PHASE BEHAVIOR AND NANOSTRUCTURE CHARACTERIZATION

Congreso: 6th EuCheMS Chemistry congress

Sevilla, del 11 al 15 de septiembre de 2016


Chemistry: Shaping the FUTURE
6th EuCheMS Chemistry Congress
Congress Program
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Environment-responsive supramolecular solvents based on the self-assembly of undecenoic acid polymers: Phase behavior and nanostructure characterization

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Presenting author: José Ángel Salatti

SUMMARY:
Tailored solvents represent a wholly new paradigm in manufacturing and processing. Designing and producing solvents that meet programmed, specific requirements for a particular purpose greatly increases our ability to improve process economics, selectivity, and yield.

Self-assembly processes are giving access to advanced functional supramolecular materials and providing an original approach to nanoscience and nanotechnology, however such appealing power remains virtually unexplored for the production of tailored solvents, although self-assembly has already proved an invaluable strategy for the energyless synthesis of ordered structure-based liquid phases from amphiphiles.

In this work, environment-responsive supramolecular solvents based on undecenoic acid polymers were synthesized and characterized. Supramolecular solvent (SUPRAS) is a relatively recent term that refers to the surfactant-rich phase (i.e. coacervate) separated from colloidal solutions of surfactants by self-assembly processes. This term emphasizes that amphiphiles form self-organized supramolecular structures in the separated liquid phase, this being the most distinctive feature compared to molecular solvents and ionic liquids.

SUPRASs were synthesized by adding water to solutions of undecenoic acid polymer in tetrahydrofuran (THF) or ethanol. Water promoted the self-assembly of the polymer and caused liquid phase separation. Phase diagrams from ternary mixtures of undecenoic acid/THF/water and undecenoic acid/ethanol/water were obtained and the boundaries for the region where separation of two isotropic phases occurred were depicted. Influence of the temperature and salts on these boundaries was investigated and the volume of SUPRAS obtained as a function of the amount of amphiphile and the percentage of water used in the synthesis was determined. The composition of the SUPRASs under different synthetic conditions was investigated and the aggregates in which the undecenoic acid polymer arranges were studied by TEM and SEM.

The global composition of the solvent, the size of the coacervate droplets that form it, and the aqueous cavities of the inverted hexagonal arrangement of the undecenoic acid polymer can be tailored by controlling the environment (specifically, the THF:water and ethanol:water ratio in the bulk solution) for self-assembly. Interestingly, SUPRAS are highly adaptive and the previous features can all be reversed by modifying the environment. The spontaneous self-assembly of these solvents followed predictable routes, and their composition and volume can be accurately predicted from equations derived in this work.

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PÓSTER NACIONAL

DISOLVENTES SUPRAMOLECULARES CONSTITUIDOS POR NANOESTRUCTURAS HEXAGONALES VOLÁTILES PARA LA SIMPLIFICACIÓN DEL TRATAMIENTO DE MUESTRAS BIOLÓGICAS

Congreso: XX Reunión de la Sociedad Española de Química Analítica

Valencia, del 1 al 3 de julio de 2015

XX Reunión de la Sociedad Española de Química Analítica

Santiago de Compostela 1-3 de julio de 2015

CERTIFICADO DE PARTICIPACIÓN

El Comité Organizador certifica que:
N. Caballero-Casero, S. Rubio

Han presentado la comunicación en formato ORAL **"DISOLVENTES SUPRAMOLECULARES: LÍQUIDOS NANOESTRUCTURADOS SENSIBLES A ESTÍMULOS AMBIENTALES PARA LA EXTRACCIÓN DE COMPUESTOS ORGÁNICOS"** en la XX Reunión de la Sociedad Española de Química Analítica, celebrada en Santiago de Compostela, del 1-3 de julio de 2015

Y para que así conste, se expide el presente certificado
En Santiago de Compostela, a 3 de julio de 2015

Dra. Pilar Bermejo Barrera
Presidenta del Comité Organizador

P22

DISOLVENTES SUPRAMOLECULARES CONSTITUIDOS POR NANOESTRUCTURAS HEXAGONALES VOLÁTILES PARA LA SIMPLIFICACIÓN DEL TRATAMIENTO DE MUESTRAS BIOLÓGICAS

José Ángel Salatti-Dorado, Noelia Caballero-Casero, Mª Dolores Sicilia, Mª Loreto Lunar y Soledad Rubio
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Bisfenol A (BPA), uno de los compuestos químicos con mayor producción a nivel mundial, se ha considerado tradicionalmente un estrógeno débil. Sin embargo, numerosos estudios in vitro realizados recientemente han demostrado que BPA puede alterar la función normal del sistema endocrino y producir perturbaciones en las funciones celulares a concentraciones tan bajas como 0.23 ng L^{-1}. El consumo de alimentos embotellados y enlatados constituye la principal vía de exposición humana a BPA ya que este compuesto se usa en la fabricación de plásticos policarbonatados y resinas epoxi. Estos materiales se utilizan como contenedores alimentarios así como recubrimiento interno de latas de conservas y bebidas. Bisfenol A migra desde estos contenedores a las bebidas y a los alimentos a concentraciones entre 0.1-3.4 ng mL^{-1} y 0.3-458 ng g^{-1}, respectivamente. Estudios recientes ponen de manifiesto que BPA está presente en un 95% de las muestras de orina humana analizadas (concentración media 0.72 ng mL^{-1}) [1], por lo tanto es de interés disponer de métodos sencillos, rápidos, económicos y respetuosos con el medio ambiente que permitan determinar bajas concentraciones de BPA en orina para evaluar la exposición humana a este contaminante.

Hasta la fecha, los métodos desarrollados para la determinación cuantitativa de BPA en orina requieren largos y complejos procedimientos para el tratamiento de la muestra que generalmente incluyen extracciones repetitivas con grandes volúmenes de disolventes orgánicos o SPE, seguido de una etapa de purificación y evaporación con nitrógeno. La cuantificación de lleva a cabo mediante cromatografía líquida con detección fluorescente o acoplada a espectrometría de masas.

En este trabajo se propone el uso de un nuevo disolvente supramolecular constituido por micelas inversas de hexanol para la extracción de BPA presente en muestras de orina humana con el objetivo de simplificar la etapa de tratamiento de muestra. El procedimiento implica la adición de 82.5 µL de hexanol y 150 µL de tetrahidrofurano a 1267 µL de orina previamente hidrolizada. A continuación se agita la mezcla durante 7 min para favorecer la incorporación del analito al disolvente supramolecular generado *in situ* y después se centrifuga para acelerar la separación de fases. Para eliminar interferencias por parte de la matriz de la muestra, 75 µL del SUPRAS formado (123 µL) conteniendo el BPA, se llevan a sequedad con nitrógeno y posteriormente el analito se disuelve con 300 µL de una mezcla metanol:agua (50:50). El extracto purificado se analiza mediante cromatografía líquida-espectrometría de masas. El límite de cuantificación del método fue 0.022 ng mL^{-1} y la precisión, expresada como desviación estándar relativa del 4,5%. La aplicabilidad del método se estudió analizando 20 muestras de orina diferentes, BPA se encontró en el 100% de las mismas a concentraciones que oscilaron entre 0,14 y 0,70 ng mL^{-1}. Las recuperaciones obtenidas para muestras adicionadas con 1 ng mL^{-1} oscilaron en el intervalo 96-110%.

[1] X. Zhou, J.P. Kramer, A.M. Calafat, X. Ye. J. Chromatogr. B (2014) 944, 152-166.

ORAL NACIONAL

SÍNTESIS Y CARACTERIZACIÓN DE FASES LÍQUIDAS NANOESTRUCTURADAS CONSTITUIDAS POR AGREGADOS ESTABLES DE MICELAS POLIMÉRICAS

Congreso: Quinta edición de encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces Reunión de la Sociedad Española de Química Analítica

Córdoba, del 5 al 6 de febrero de 2015

V Workshop

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LIBRO DE RESÚMENES

NANOUCO V

Encuentro sobre Nanociencia y Nanotecnología
de Investigadores y Tecnólogos Andaluces

Córdoba, 5 y 6 de Febrero de 2015

O18

SÍNTESIS Y CARACTERIZACIÓN DE FASES LÍQUIDAS NANOESTRUCTURADAS CONSTITUIDAS POR AGREGADOS ESTABLES DE MICELAS POLIMÉRICAS

José Ángel Salatti Dorado, Francisco José López Jiménez, Soledad Rubio
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El desarrollo de disolventes alternativos a los disolventes orgánicos ha despertado un enorme interés en los ámbitos industrial y científico, sectores en los que se deben atender las exigencias de las políticas medioambientales y sociales (ej. normativa europea REACH) y, al mismo tiempo, desarrollar materiales con propiedades adecuadas para su uso en procesos, aplicaciones y diseños innovadores en un mundo cada vez más tecnológico. Aunque se han desarrollado aplicaciones innovadoras utilizando fluidos supercríticos y líquidos iónicos, sus prestaciones no permiten sustituir a los disolventes orgánicos a gran escala y los costes de producción son, en la mayoría de los casos, inasumibles. Por tanto, es fundamental investigar nuevas alternativas que den respuesta a los retos planteados.

El autoensamblaje y coacervación de compuestos anfifílicos ofrece infinitas posibilidades para el diseño de fases líquidas nanoestructuradas. El tamaño, morfología y funcionalidad de los agregados que constituyen estas fases puede controlarse seleccionando la estructura y concentración de las moléculas anfifílicas y el ambiente en el que se produce el autoensamblaje y por lo tanto la coacervación constituyen una excelente vía para la obtención de disolventes funcionales. Estos disolventes poseen propiedades intrínsecas y operacionales excepcionales para su utilización en procesos de extracción.[1,2] Sin embargo, ya que la nanoestructuras de estas fases líquidas están integradas por moléculas unidas mediante enlaces no covalentes, el coacervado se solubiliza en agua o disoluciones hidro-orgánicas si las condiciones experimentales de coacervación no se mantienen constantes en la disolución de equilibrio, lo cual requiere a veces pH extremos, altas temperaturas, la adición de elevadas concentraciones de sal, etc. Este hecho ha reducido su aplicabilidad en procesos de extracción donde están implicados grandes volúmenes de agua.

En este trabajo se propone la coacervación de micelas poliméricas de poli-(10-undecilenato) de sodio (poli-SUD), inducida por cloruro de tetrahexilamonio (THACl), para la producción de fases líquidas nanoestructuradas constituidas por agregados estables. Los coacervados así producidos son inmiscibles en disoluciones acuosas con composición diferente a la de la disolución de equilibrio resultante del proceso de autoensamblaje. Se han estudiado los diagramas de fases en función de la cantidad de poli-SUD y THACl así como la influencia en los mismos del tipo de sal de amonio cuaternario, el pH, la temperatura y sales inorgánicas. Se ha derivado una ecuación que permite predecir el volumen de disolvente obtenido en función de la cantidad de poli-SUD and THACl usados para el autoensamblaje. Asimismo se ha determinado la composición química del disolvente y se han caracterizado las nanoestructuras que lo integran. La baja solubilidad de estos disolventes en agua permite su aplicación a la extracción de contaminantes traza, con elevados factores de preconcentración, y al tratamiento de aguas residuales.

(1) Ballesteros-Gómez, A.; Sicilia, D.; Rubio, S. *Anal. Chim. Acta* **2010**, *677*, 108-130
(2) López-Jiménez, F.J.; Rubio, S.; Pérez-Bendito, D. *J. Chromatogr. A*, **2008**, *1195*, 25-33

ORAL NACIONAL

SÍNTESIS Y CARACTERIZACIÓN DE SISTEMAS SUPRAMOLECULARES CONSTITUIDOS POR AGREGADOS ESTABLES. APLICACIÓN EN PROCESOS DE EXTRACCIÓN ANALÍTICA

Congreso: III Congreso Científico de Investigadores en Formación en Agroalimentación

Córdoba, del 18 al 19 de noviembre de 2014

La Coordinadora Académica del Campus de Excelencia Internacional en Agroalimentación ceiA3

ACREDITA que :

JOSÉ ANGEL SALATTI DORADO

ha presentado **la COMUNICACIÓN ORAL** que lleva por título :

SÍNTESIS Y CARACTERIZACIÓN DE SISTEMAS SUPRAMOLECULARES CONSTITUIDOS POR AGREGADOS ESTABLES. APLICACIÓN EN PROCESOS DE EXTRACCIÓN ANALÍTICA.

en el **III Congreso Científico de Investigadores en Formación en Agroalimentación ceiA3,** organizado por la Escuela Internacional de Doctorado en Agroalimentación eidA3, celebrado en Córdoba los días 18 y 19 de noviembre de 2014.

Y para que así conste, se expide y firma este certificado en Córdoba, a 19 de noviembre de 2014

Fdo: JULIETA MÉRIDA GARCÍA

Coordinadora Académica del ceiA3

Rectorado de la Universidad de Córdoba – Avd. Medina Azahara 5, 14071 CÓRDOBA

José Ángel Salatti Dorado

Título completo de la tesis:

Síntesis y caracterización de sistemas supramoleculares constituidos por agregados estables. Aplicación en procesos de extracción analítica.

Objetivos previstos de la Tesis:

El objetivo general de la Tesis Doctoral es aprovechar los últimos avances que se han producido en Química Supramolecular relacionados con el autoensamblaje, así como la experiencia de nuestro grupo de investigación en este ámbito, para diseñar disolventes y sólidos que se adapten a los principios de la química verde, tengan la capacidad de mejorar los procesos de extracción tanto desde el punto de vista operacional como de rendimiento, y permitan el desarrollo de aplicaciones innovadoras en extracciones analíticas.

Los objetivos específicos de este proyecto son:

1. Diseño, síntesis y caracterización de extractantes supramoleculares constituidos por agregados estables

1.1. Disolventes supramoleculares

Se sintetizarán micelas poliméricas de poli(undecilenato de sodio) y poli(undecenilsulfato de sodio) mediante polimerización radicálica en diferentes posiciones de la cadena hidrocarbonada del tensioactivo, y se estudiará el autoensamblaje y coacervación de las mismas utilizando sales de amonio cuaternario. Se investigará la estructura de todos los disolventes sintetizados y se estudiarán sus propiedades físico-químicas.

1.2. Admicelas

Se sintetizarán admicelas mediante quimiadsorción a partir de las micelas poliméricas señaladas en el apartado anterior y nanopartículas magnéticas de magnetita. Se determinarán las isotermas de adsorción, se investigará la estructura del material adsorbente resultante y se estudiarán sus propiedades físico-químicas.

2. Desarrollo de nuevos formatos en extracción supramolecular

2.1. Extracción en fase sólida con disolventes supramoleculares

Partiendo de la hipótesis de que los disolventes supramoleculares sintetizados serán inmiscibles en agua y mostrarán fuerte adhesión a materiales sólidos, se investigará su uso en formato SPE para la extracción de solutos en la fase líquida superficial constituida por las micelas poliméricas coacervadas adsorbidas a diferentes soportes. Se pretende proporcionar nuevos materiales para SPE que ofrezcan mecanismos mixtos para la solubilización de los solutos.

2.2. Extracción magnética supramolecular

El uso de nanopartículas magnéticas en procesos de extracción en fase sólida tiene como ventaja la fácil manipulación de las mismas mediante un imán. La quimioadsorción de micelas poliméricas sobre estas partículas producirá un adsorbente con capacidad para establecer diferentes tipos de enlace (iónicos, puentes de hidrógeno, Van der Waals, etc) con los solutos. Se realizará un amplio estudio para determinar los parámetros operacionales generales que afectan a la extracción supramolecular y la capacidad de estos adsorbentes para la extracción de solutos en un amplio intervalo de polaridad.

3. Desarrollo de estrategias innovadoras para la microextracción de contaminantes emergentes

3.1. Microextracción de contaminantes emergentes en análisis de alimentos

Los materiales supramoleculares desarrollados se aplicarán a la extracción de compuestos con actividad estrogénica en muestras de alimentos y bebidas enlatadas, dado el interés actual por determinar el nivel de exposición humana a este tipo de contaminantes. Se prestará especial atención a la extracción de multiresiduos y los métodos desarrollados se evaluarán siguiendo los criterios establecidos en la decisión europea 2002/657/EC.

3.2. Microextracción de contaminantes emergentes en análisis biológico

Se desarrollarán metodologías innovadoras para la extracción de compuestos con actividad estrogénica en orina y suero. La presencia de estos compuestos en fluidos biológicos humanos es ubicua y existe gran interés en la evaluación de la exposición a los mismos a través de estudios epidemiológicos, lo que requiere el procesamiento de un número elevado de muestras, y por tanto de métodos simples, rápidos y económicos.

Método de trabajo:

OBJETIVO 1. Diseño, síntesis y caracterización de extractantes supramoleculares constituidos por agregados estables

La metodología general que seguiremos consistirá en: (1) revisión bibliográfica y discusión crítica de los antecedentes; (2) selección del tensioactivo y condiciones ambientales adecuadas para generar el material supramolecular con las propiedades y funciones requeridas; (3) síntesis de las micelas poliméricas mediante las reacciones de polimerización radicálica descritas en la bibliografía; (4) síntesis de los disolventes supramoleculares utilizando procesos de autoensamblaje; (5) síntesis de las admicelas mediante quimiadsorción utilizando los procedimientos previamente descritos por nuestro grupo de investigación; (6) estudio de las propiedades físico-químicas generales de los mismos así como de las propiedades singulares para las que fueron programados; (7) caracterización estructural de los materiales y determinación del tamaño y polidispersión de los agregados que los constituyen.

Los agentes coacervantes que se utilizarán para la formación de los Disolventes Supramoleculares serán las sales de alquil amonio (tetraetil, tetrapentil y tetrahexil) a diferentes valores de pH.

La polimerización de las micelas se llevará a cabo en las siguientes posiciones: en el extremo terminal de las cadenas hidrocarbonadas para producir moléculas "tipo estrella" (star-type molecules); en la interfase núcleo-corona o en la corona, para crear una nano-membrana rodeada de un núcleo de cadenas lineales; o en la superficie.

La caracterización de los materiales supramoleculares se llevará a cabo mediante microscopía óptica, electrónica (de barrido, SEM, y de transmisión, TEM) y microscopía de fuerza atómica. Asimismo, se utilizará difracción de rayos X y técnicas de dispersión de luz.

OBJETIVO 2. Desarrollo de nuevos formatos en extracción supramolecular

La metodología general que seguiremos consistirá en: (1) revisión bibliográfica y discusión crítica de los antecedentes; (2) estudio de diferentes materiales soporte para la adsorción de los disolventes supramoleculares; (3) estudio de los parámetros operacionales que afectan a la extracción en fase sólida con disolventes supramoleculares y extracción magnética supramolecular; (4) estudio de la capacidad de estos adsorbentes para la extracción de solutos en un amplio intervalo de polaridad para determinar su potencial en el análisis de multiresiduos; (5) estudio de la capacidad de adsorción de los principales componentes de muestras agroalimentarias, biológicas y ambientales (ej. proteínas, carbohidratos, lípidos, ácidos húmicos, etc) para predecir la selectividad de los procesos de extracción.

Se ensayarán solutos con muy diferentes propiedades físico-químicas, estructurales y funcionales (micotoxinas, drogas, compuestos con actividad estrogénica, tensioactivos, etc) cuyo análisis es interesante por las consecuencias económicas, biológicas y/o sociales que se derivan de su toxicidad. Las técnicas analíticas empleadas serán principalmente cromatografía de líquidos con detección fluorimétrica y de diodos en fila.

OBJETIVO 3. Desarrollo de estrategias innovadoras para la microextracción de contaminantes emergentes.

La metodología general que seguiremos consistirá en: (1) revisión bibliográfica y discusión crítica de los antecedentes relacionados con la extracción de sustancias con actividad estrogénica en muestras de alimentos y fluidos biológicos; (2) selección de los compuestos objeto de estudio; (3) determinación de las propiedades extractivas de los materiales supramoleculares desarrollados (recuperaciones, factores de concentración, selectividad, etc.) para los solutos y aplicaciones de interés; (4) desarrollo de las correspondientes metodologías de extracción en el formato idóneo para cada aplicación; (5) validación de los métodos analíticos desarrollados de acuerdo a los criterios establecidos en la decisión europea 2002/657 EC y la guía para validación de métodos bioanalíticos (European Medicines Agency, 2011) para la determinación de contaminantes en alimentos y bebidas enlatadas y fluidos biológicos (orina y suero), respectivamente; (6) aplicación de los métodos desarrollados a estudios epidemiológicos.

Las técnicas analíticas empleadas serán principalmente cromatografía de líquidos, micro o convencional, acoplada a espectrometría de masas con detector de trampa iónica y triple cuadrupolo-trampa iónica. Para estudios epidemiológicos, seguiremos con la colaboración del grupo de investigación de la Dra. Vrijheid (Fundacio Centre de Recerca en Epidemiologia Ambiental, Barcelona).

Aportaciones:

Las investigaciones que se proponen en este Proyecto de Tesis Doctoral tienen como finalidad el desarrollo, caracterización y aplicación en procesos de extracción de nuevos materiales nanoestructurados producidos a partir de sustancias anfifílicas utilizando el autoensamblaje como ruta sintética.

De forma general se espera las siguientes contribuciones:

• Avances en el conocimiento de los procesos de autoensamblaje de los compuestos anfifílicos para producir materiales en fase sólida y líquida, principalmente relacionados con la influencia de las condiciones ambientales y la estructura del tensioactivo en la naturaleza física y macroestructura del material supramolecular resultante, así como en la microestructrura de los agregados que los constituyen.

• Desarrollo de materiales supramoleculares con propiedades y funciones específicas previamente programadas. El estudio del grado de control que las condiciones ambientales puedan ejercer sobre las propiedades y funciones de estos materiales proporcionará conocimientos para avanzar en la obtención de materiales supramoleculares a la carta.

• Mejora de la tecnología de preparación de muestras en la determinación de compuestos con actividad estrogénica, en análisis de alimentos y biológico.

Las principales actividades del programa formativo asociado al proyecto son:

a) Búsqueda y discusión critica de la bibliografía científica relacionada con los tópicos de interés para el proyecto.

b) Realización de diferentes cursos relacionados con el manejo de las técnicas e interpretación de los resultados obtenidos para la caracterización de materiales.

c) Publicación de un mínimo de dos artículos por año en revistas de alto índice de impacto, con presentación previa o paralela en al menos un congreso científico de alto nivel.

d) Asistencia y presentación de contribuciones científicas, con preferencia mediante comunicación oral, en congresos nacionales e internacionales relacionados con la temática del proyecto.

e) Estancia en un centro de excelencia europeo (3-6 meses) para el desarrollo de actividades que complementen la formación investigadora del solicitante.

f) Presentación y defensa de la Tesis Doctoral en la modalidad de Mención Internacional.